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The Site of Covalent Attachment in the Crystalline Osmium-tRNA^{fMet} Isomorphous Derivative†

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ABSTRACT: The site of osmium attachment in the crystalline isomorphous derivative of yeast tRNA^{fMet} is shown to be cytidine-38, the first hydrogen-bonded base to the 3′ side of the anticodon loop. The site of modification was determined chromatographically from nuclease digests of dissolved crystals of ^{185,191}Os-tRNA^{fMet}. A new and potentially useful column packing, Al-Pellionex-WAX, was employed in this analysis. The osmium adduct includes two pyridine molecules, is unusually stable, and does not interfere with enzymatic aminoacylation. Having established the attachment site, the osmium atom becomes a heavy-atom marker of a specific residue to aid in the interpretation of the electron-density map of yeast tRNA^{fMet}.

An osmium derivative of crystalline formylmethionine transfer RNA from yeast (yeast tRNA^{fMet})[1] has previously been characterized crystallographically (Schevitz *et al.*, 1972) and was shown to have a single dominant osmium site. We report here that the osmium is bound to the cytidine at position 38 in the sequence, the first hydrogen-bonded residue of the anticodon loop (Simsek and RajBhandary, 1972). These experiments were carried out primarily to facilitate the molecular structure determination of yeast tRNA^{fMet} by correlating the position of the osmium in the electron-density map as determined crystallographically, with the position of a specific residue in the sequence, thereby providing a guide point for tracing the polynucleotide chain. Evidence is also presented demonstrating that the addition of osmium to the molecule does not alter the ability of the yeast initiator tRNA to be aminoacylated by methionine:tRNA ligase, indicating that the site of osmium attachment does not interfere with the formation of a productive tRNA–ligase complex.

The original work of Criegee demonstrating that nitrogen heterocycles enhance the stability of osmate esters (Criegee *et al.*, 1942) has been utilized by a number of workers seeking to attach heavy atoms to nucleic acids for structural studies (Beer *et al.*, 1966; Burton and Riley, 1966). Recently, detailed investigations by Behrman and coworkers (Subbaraman *et al.*, 1971) have shown that relatively stable osmium adducts of pyrimidines may be formed in the presence of pyridine. We show here that exposure of crystals of yeast tRNA^{fMet} to a mother liquor containing Os(VI) and pyridine produced a derivative containing approximately 1 atom of osmium and two molecules of pyridine for each molecule of tRNA. The adduct is stable and cannot be reversed by conditions known to disrupt the secondary and tertiary structure of the tRNA molecule. Although the osmium and pyridines are also irreversibly bound to a dodecamer released by ribonuclease T_1, a certain degree of three-dimensional structural integrity is apparently required, since complete digestion of this fragment disrupts the adduct.

We also present two new and convenient techniques developed in the course of this work to analyze and isolate nuclease-digest products of subnanomolar quantities of tRNA.

Materials and Methods

Yeast tRNA^{fMet}. tRNA^{fMet} was purified from bulk yeast tRNA prepared according to the method of Holley (1963), using the purification scheme of Johnson *et al.* (1970) as summarized by Pasek *et al.* (1973). Crystals were grown as described by Johnson *et al.* (1970) from preparations of tRNA^{fMet} which accepted 1.8 nmol of methionine/A_{258} unit. Acceptance levels as low as 1.4 nmol/A_{258} unit were encountered but only in crystals which had been irradiated.

Preparation of Os-tRNA^{fMet}. Crystals of yeast tRNA^{fMet} were soaked in a stabilizing supernatant solution containing potassium osmate and pyridine as described by Schevitz *et al.* (1972). This "soak solution" was obtained by a 50-fold dilution of a 1 M $K_2OsO_2(OH)_4$, 3.1 M pyridine "stock solution," prepared by adding an equal volume of a 1:1 (vol.) mixture of pyridine and 1 N HCl to a 0.2 M aqueous suspension of $K_2OsO_2(OH)_4$ (Alfa Inorganics). This "stock

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[1] Abbreviations used are: yeast tRNA^{fMet}, formylatable methionine tRNA from yeast; A_{258} unit, that amount of tRNA when dissolved in 1 ml of H_2O produces an absorbance of 1 at 258 nm with a 1-cm light path.

solution" was heated briefly to solubilize any precipitate before dilution with the crystal stabilizing solution. No precipitate formed in the pale amber "soak solution" which was 0.002 M in osmium and 0.063 M in pyridine.

Preparation of $[^{185,191}Os]K_2OsO_2(OH)_4$. The radioactive osmium derivative of yeast tRNA^{fMet} was made by using labeled $K_2OsO_2(OH)_4$ prepared from an isotopic mixture of ^{185}Os and ^{191}Os as hexachlorosmic acid ($[^{185,191}Os]H_2OsCl_6$, Amersham-Searle).

Typically, an equal volume of 10 N NaOH was added to 0.14 mmol of $[^{185,191}Os]H_2OsCl_6$ (Amersham-Searle, 2.1 Ci ^{185}Os, 17.2 Ci of ^{191}Os/mol) in 7 ml of 1.7 N HCl. The greenish-yellow solution of hexachlorosmic acid initially became colorless, then over a period of several days at room temperature, the solution became purple, then black. Low-speed centrifugation yielded a granular black powder, presumably OsO_2. The powder was washed twice with 5 ml of 1 N NaOH and once with water and suspended in 0.7 ml of cyclohexane. To the suspension was added 2 ml of freshly prepared aqueous 0.3 M $NaIO_4$. The two phases were then mixed until all of the yellowish-brown color of OsO_4 appeared in the organic phase. (It was occasionally necessary to add a small amount of HCl to neutralize any residual NaOH to prevent the formation of the red-colored perosmate, $[OsO_4(OH)_2]^{2-}$, which is water soluble.) The cyclohexane phase, containing the OsO_4, was removed and the osmium reduced by the addition of 0.4 ml of an aqueous solution containing 0.4 mmol of KOH and 0.7 mmol of ethanol. The resulting pink precipitate of potassium osmate was left to develop in the cold. The $K_2OsO_2(OH)_4$ was centrifuged and the pellet washed with cold absolute ethanol and anhydrous ethyl ether. The rose-colored powder was dried under vacuum at room temperature. The yields of $[^{185,191}Os]K_2OsO_2(OH)_4$ ranged from 50 to 85%. The osmium radioisotopes were counted in a Nuclear-Chicago well-counter. The molar specific radioactivity of the 0.1 M $K_2OsO_2(OH)_4$ "stock solution" was determined to an accuracy of ±10% by measuring the concentration of the osmium spectrophotometrically as the thiourea complex (Dwyer and Gibson, 1951).

Preparation of $^{185,191}Os$ + $[^{14}C]$*Pyridine Soak Solution.* Crystals of yeast tRNA^{fMet} were also soaked in a stabilizing supernatant containing [^{14}C]pyridine as well as $^{185,191}Os$. The composition of this soak solution was as described in the preparation of Os-tRNA^{fMet} except that [^{14}C]pyridine (ICN, 12.9 Ci/mol, 0.85 M in H_2O) was used at a final radiospecific activity of 1.75 Ci/mol.

Enzymatic Digests. RNase T_1 (Calbiochem) was dissolved on 0.02 M Tris-HCl (pH 7.5) to a concentration of 5000 units/ml and twice heated to 85° for 2 min to inactivate any phosphatase contaminants (Reeves *et al.*, 1968). Typically, a solution of 2 A_{258} units of tRNA^{fMet} (or the Os adduct) was dialyzed for three 2-hr periods against 1 l. of H_2O, lyophilized, dissolved in 0.12 ml of 0.1 M Tris-HCl (pH 7.5) and incubated for 1 hr at 37° with 50 units of RNase T_1.

Pancreatic RNase (Sigma, RNase A 5× crystallized) was dissolved in 0.02 M Tris-HCl (pH 7.5) to a concentration of 0.5 mg/ml; 0.1 A_{260} unit of lyophilized oligonucleotide was dissolved in 50 μl of 0.1 M Tris-HCl (pH 7.5) and digested with 2.5 μg of pancreatic RNase for 1 hr at 37°.

Oligonucleotides were enzymatically degraded to nucleosides by adding to the polynucleotide solution 0.1 volume of a solution containing 0.25 mg/ml each of pancreatic RNase, *E. coli* alkaline phosphatase (Worthington, BAPF), and snake venom phosphodiesterase (Worthington) in 0.01 M $MgCl_2$–0.1 M Tris-HCl (pH 8.0). Typically 0.05 A_{260} unit of lyophilized oligonucleotide was dissolved in 50 μl of 0.01 M $MgCl_2$–0.1 M Tris-HCl (pH 8.0) and digested with 5 μl of the mixture of nucleases for 3 hr at 37°.

Column Chromatography. Oligonucleotide fragments from RNase T_1 and pancreatic RNase digests were separated by one of three methods: (a) on DEAE-cellulose (Schleicher and Schuell) in the presence of urea (Tomlinson and Tener, 1963), (b) on Bio-Gel P-6 (Bio-Rad) in 0.02 M Tris-HCl (pH 7.8) or (c) on Al-Pellionex-WAX (Reeve-Angel). Pooled fractions from DEAE-cellulose and Bio-Gel columns were rid of salt and urea by absorption of the oligonucleotide to a 0.3 ml column of DEAE-cellulose, elution with 2 M triethylammonium bicarbonate (pH 8) (Rushizky and Sober, 1962), and lyophilized. Fractions from Pellionex columns were desalted on a 1 × 30 cm column of Bio-Gel P-2 in 0.02 M NH_4HCO_3 and lyophilized.

Nucleoside analyses were performed on Aminex A-6 (Bio-Rad) essentially according to Uziel *et al.* (1968). Samples were injected onto the column through a slider valve (Chromatronix) nominally containing 20 μl.

All column effluents were continuously monitored at 254 nm (Altex, Model 154 or Chromatronix, Model 220). Quantitation of nucleoside analyses was made by the "Xerox-cut-and-weigh" technique standardized by applying known amounts of nucleoside to the column. Typically, noise levels were approximately 1.0×10^{-4} A_{258} unit. Accuracy was estimated to be ±5% for guanosine and adenosine and ±10% for cytidine and uridine.

Attempts to Disrupt the Osmium Adduct. The integrity of the osmium adduct was tested by incubating radioactive Os-tRNA^{fMet} or Os-oligonucleotide under denaturation conditions with reagents which might compete with nucleic acid for the binding of osmium. Samples of approximately 1 nmol of Os-tRNA^{fMet} or Os-oligonucleotide were dissolved in 20-μl solutions of 0.1 M NaCl, 0.01 M sodium cacodylate, 0.01 M Na_2EDTA, 33% formamide, and containing at least a 100-fold excess of competing reagent. The solutions were incubated at 50° for 10 hr, then chromatographed at 50° on a 0.3 × 100 cm column of G-25 Sephadex (Pharmacia) in 0.1 M NaCl, 0.01 M sodium cacodylate, 0.001 M Na_2EDTA, and 33% formamide. The periodate treatment of the osmium adduct which led to release of the Os on P-2 gel filtration was carried out with a 100-fold molar excess of $NaIO_4$ (typically 5m*M*) at 50° for 1 hr.

Aminoacylation of Yeast tRNA^{fMet} and Os-tRNA^{fMet}. A crude preparation methionine:tRNA ligase from *E. coli*, prepared according to Muench and Berg (1966), was used to routinely measure the amino acid acceptance of both yeast tRNA^{fMet} and Os-tRNA^{fMet}. The possible disruption of the osmium adduct by enzymatic aminoacylation was investigated by adsorbing the methionyl-Os-tRNA^{fMet} to a 1-ml column of DEAE-cellulose, washing with 0.3 M NaCl to remove all unbound radioactivity, and eluting the aminoacyl-tRNA with 1 M NaCl. Aliquots of the eluate were counted for [^{14}C]methionine and $^{185,191}Os$.

A partially purified preparation of yeast methionine: tRNA ligase (gift of Margaret D. Rosa) was used to obtain the time course of the aminoacylation reaction.

Results

Stoichiometry. As previously described (Schevitz *et al.*, 1972), crystals of yeast tRNA^{fMet} reacted with osmium

TABLE I: Stoichiometry of Osmium and Pyridine.

Sample	A_{258} Units	Radioactivity		g-atoms of Osmium[c]	mol of Pyridine
		Osmium[a]	Pyridine[b]	mol of tRNA	mol of tRNA
$tRNA^{fMet}$ [d]	2.1	7	30	0.0	0.0
Os-$tRNA^{fMet}$ [d,e]	2.0	4051		0.9	
	5.2	8128		1.0	
	3.3	1976		0.9	
Os-$tRNA^{fMet}$ [d]	4.1	2649	9432	0.9	2.3
Os-$tRNA^{fMet}$ [d,f]	1.9	1368	4040	1.0	2.1
Methionyl-Os-$tRNA^{fMet}$ [d,g]	1.2	2325		0.9	

[a] CPM ^{185,191}Os corrected for decay. [b] CPM ^{14}C counted in Aquasol (New England Nuclear) and corrected for "spill over" of Os radioactivity. [c] A_{258} taken as 2.0 nmol. [d] All crystals were dissolved; then the solution was desalted on G-25 Sephadex. [e] Three separate preparations of crystalline ^{185,191}Os-bipyridyl adduct of yeast $tRNA^{fMet}$. [f] Crystals equilibrated with supernatant solution containing nonradioactive Os(VI) and pyridine. [g] Os-$tRNA^{fMet}$ was enzymatically methionylated with a crude prep of *E. coli* enzymes (Muench and Berg, 1966) to a maximum level of 1.5 nmol of Met/A_{258} unit. The reaction mixture was processed on DEAE-cellulose as described in the Materials and Methods section.

gradually became colored, and appeared blue when viewed down the hexagonal axis and amber when viewed normal to this axis. This color change was complete in about 3 weeks. The stoichiometry of the osmium-$tRNA^{fMet}$ adduct was determined after G-25 Sephadex chromatography of dissolved crystals either by neutron activation analysis (Schevitz *et al.*, 1972) or by the use of radioactive osmium (Table I). By either method, approximately 1.0 (+10%) g atom of osmium was found irreversibly bound/mol of $tRNA^{fMet}$. The same stoichiometry was found after Os-$tRNA^{fMet}$ was subjected to solvent and temperature conditions known to disrupt the secondary structure. Crystals reacted with ^{185,191}Os and [^{14}C]pyridine were found to contain 2.2 (±5%) mol of pyridine and 1.0 (±10%) g atoms of Os/mol of tRNA after G-25 Sephadex chromatography.

The Stability of the Osmium Adduct. The stability of the osmium adduct in both $tRNA^{fMet}$ and the dodecanucleotide fragment, fragment 12′ (see below), was investigated by denaturation experiments as described in the Materials and Methods section. Both ^{185,191}Os-$tRNA^{fMet}$ and fragment 12′ were challenged with excess amounts of pyridine, thymidine, cytidine, ApC, ApApC, ethylene glycol, or saturated sodium carbonate, under conditions of solvent and temperature known to disrupt secondary structure. In all cases the osmium reproducibly cochromatographed with the tRNA or dodecanucleotide fragment. Sodium periodate, known to oxidize osmium to Os(VIII), was required for the release of the ^{185,191}Os from the nucleic acid under the vigorous conditions described in the Materials and Methods section. The stability of the adduct is striking since the osmium bipyridyl addition products previously reported have been relatively labile (Subbaraman *et al.*, 1971; Beer *et al.*, 1966; Burton and Riley, 1966). The radioactive pyridine moieties did not exchange when crystals were soaked in a stabilizing supernatant solution containing pyridine (Table I) nor when the osmated dodecanucleotide, fragment 12′, was subjected to molecular sieving on a Bio-Gel P-6 column previously equilibrated with pyridine. No further attempts to dislodge the pyridine were made.

Determination of RNase T_1 Fragment Bearing Osmium. To establish the point of osmium attachment both $tRNA^{fMet}$ and Os-$tRNA^{fMet}$ were digested to completion with RNase T_1. DEAE-cellulose chromatographic elution profiles of these digests are shown in Figure 1a. An absence (or near absence) of fragment 12 and the elution of a new peak containing all of the radioactive Os (fragment 12′) were the only changes found in ten separate experiments. In partially reacted specimens the relative amount of fragment 12′ was proportional to the extent of osmium addition and was associated with a complementary diminution in the amount of fragment 12. For one-to-one stoichiometry of Os to $tRNA^{fMet}$, fragment 12 was completely replaced by fragment 12′. In a number of chromatograms, however, an unusual and variable degree of retardation of the osmium and fragment 12′ was noted and, in certain instances, neither the Os nor fragment 12′ could be eluted at all. To circumvent this problem subsequent separations of the products of RNase T_1 digestions were performed on columns of Bio-Gel P-6 (Figure 1b). In these experiments the recovery of Os radioactivity in the front-running peak was quantitative. This technique proved to be the most convenient and reproducible method of isolating the Os-bearing fragment.

The identity of DEAE-cellulose fragment 12 was determined by complete enzymatic digestion of the oligonucleotide to nucleosides and chromotography on Aminex A-6 (Figure 2 and Table II). The only portion of the sequence of yeast $tRNA^{fMet}$ which corresponds to the composition of fragment 12 is C_{31}-U-C-A-U-t^6A-A-C-C-C-U-G_{42}p, containing the anticodon (Figure 3; Simsek and RajBhandary, 1972) Table II also shows that the front-running Bio-Gel P-6 peak and peak 15 of the Pellionex column (described below) are identical with fragment 12 as isolated on DEAE-cellulose.[2]

Analysis of fragment 12′ (Table II) showed that this fragment has the same nucleotide composition as fragment 12, demonstrating that fragment 12′ differs from fragment 12 by only the addition of osmium. Complete nuclease digestion of fragment 12′ causes the release of the osmium suggesting that a covalently constrained conformation of nucleotide residues is required to stabilize the osmium adduct (see Discussion).

To confirm that the osmium attachment site(s) is (are)

[2] Throughout this paper, the oligonucleotide C-U-C-A-U-t^6A-A-C-C-C-U-Gp will be referred to as fragment 12 regardless of its chromatographic origin.

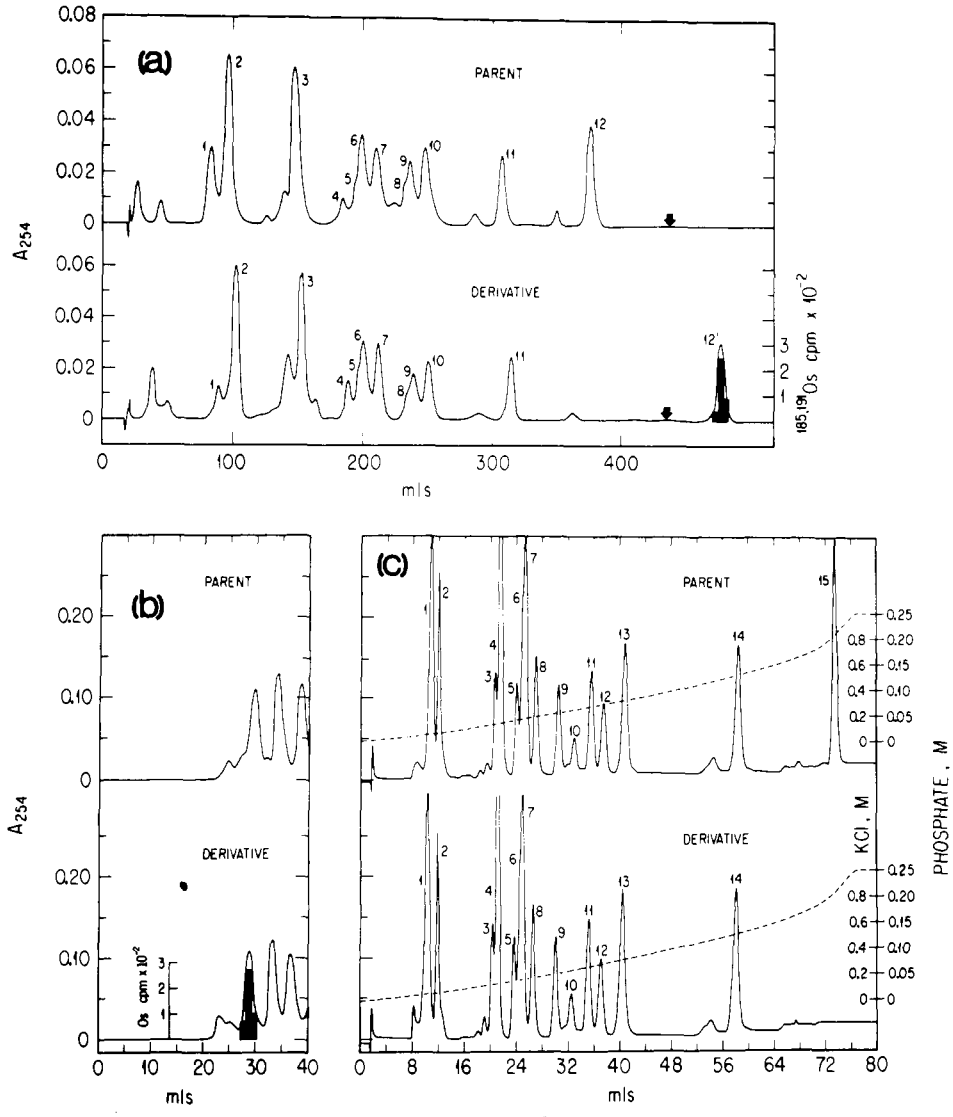

FIGURE 1: RNase T_1 digestions of 2 A_{258} units of tRNA^{fMet} and 2 A_{258} units of ^{185,191}Os-tRNA^{fMet}. (a) Chromatography on a 0.6 × 120 cm DEAE-cellulose column in the presence of 7.0 M urea and 0.02 M Tris-HCl (pH 7.5) using an 860-ml linear gradient from 0.0 to 0.45 M NaCl at 15 ml/hr. At the arrow, the column was left for 24 hr before starting a 250-ml gradient of NaCl from 0.4 to 1.0 M in 7.0 M urea–0.02 M Tris-HCl (pH 7.5). (b) Chromatography on a 0.6 × 210 cm Bio-Gel P-6 column in 0.02 M Tris-HCl (pH 7.8) at 4.5 ml/hr; only the first three peaks eluted are shown. (c) Chromatography on a 0.2 × 100 cm column of Al-Pellionex-WAX at 12.0 ml/hr (~120 psig) and maintained at 50°. A 75-ml concave gradient (0.001 M KH_2PO_4) (pH 3.4) to 0.25 M KH_2PO_4 and 1.0 M KCl (pH 3.0) started with 50 ml of 0.001 M KH_2PO_4 initially in the mixing chamber. Peak 15 is equivalent to fragment 12 of profile (a). The osmated fragment does not elute from Al-Pellionex-WAX under the conditions used.

restricted to the dodecamer, C_{31} . . . G_{42}, digests of parent and derivative tRNA were compared by chromatography on Pellionex allowing complete resolution of nearly all of the fragments (Figure 1c). As was the case for DEAE-cellulose, the chromatograms differ only with regard to fragment 12 (peak 15). Moreover, analysis of the composition of the individual fragments (Table III) indicates that there are no covalently bonded Os atoms—indeed no noticeable modifications—outside of the sequence C_{31} . . . G_{42}.

Osmium Protects a Single Cytidine from Bisulfite Catalyzed C → U Conversion. The studies on model compounds done by Behrman and coworkers (Subbaraman *et al.*, 1971, 1973) have demonstrated that, in the absence of reactive cis diols (Criegee *et al.*, 1942), pyrimidines are much more susceptible to attack by osmium than are purines and form a saturated diester across the 5,6 double bond.

Shapiro *et al.*, (1970) and Hayatsu *et al.* (1970) have shown that HSO_3^- will quantitatively convert cytidine residues to uridine with the reaction proceeding through an in-

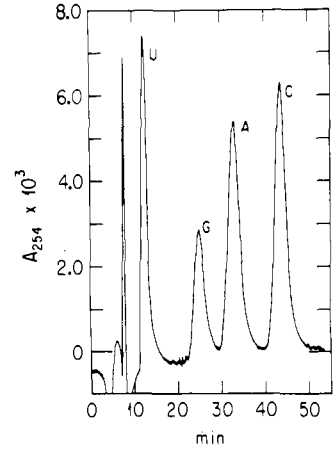

FIGURE 2: A typical nucleoside analysis (here of fragment 12) by Aminex A-6 chromatography. Approximately 100 pmol was applied to a 0.3 × 50 cm column and eluted at 50° by 0.4 M NH_4-formate (pH 4.65) at 12.0 ml/hr and ~140 psig.

TABLE II: Nucleoside Compositions of Fragments 12 and 12′.

		Relative Composition[a]				
		DEAE-cellulose		Bio-Gel P-6		Pellionex
Nucleoside	Expected[c]	Fragment 12	Fragment 12′	Fragment 12	Fragment 12′	Fragment 12
U	3	2.9 (260)	3.0 (245)	2.8 (140)	2.8 (305)	2.9 (595)
G	1	1.0 (90)	1.0 (80)	1.0 (50)	1.0 (110)	1.0 (205)
A	2	2.1 (190)	2.1 (110)	1.9 (95)	2.1 (230)	2.0 (415)
C	5	5.2 (475)	5.1 (410)	4.9 (245)	5.2 (575)	4.9 (995)
t^6A[b]	1					

[a] Compositions are relative guanosine. The numbers in parentheses are the number of pmoles found by nucleoside analysis on Aminex A-6 as described in the Materials and Methods section and Figure 3. Amounts of U and C are ±10%, G and A ±5%. [b] t^6A could not be determined since it cochromatographed with the nucleases. [c] Sequence by Simsek and RajBhandary (1972): C-U-C-A-U-t^6A-A-C-C-C-U-Gp.

TABLE III: Assignment of Fragments from Pellionex Chromatography of RNase T_1 Digestion of $tRNA^{fMet}$.

	pmol of Nucleoside Found[a]					Composition					
Peak	Urd	Guo	Ado	Cyd	N[b]	Urd	Guo	Ado	Cyd	N	Assignment
1		550					1.0				Gp
2				110	√				1.0	√	Cpm_2^2Gp
3		150			√		1.0			√	DpGp
4		135		130			1.0		1.0		CpGp
5[c]											CpCpGp[c]
6[d]											$CpUpApCpCpA_{OH}$[d]
7[c]											CpApGp[c]
8		210	405				1.0	1.9			ApApGp
9					√, √					√, √	*Ap*Gp
10	175				√, √	1.0				√, √	Upm^1Gpm^2Gp
11	125	135	140	135		0.9	1.0	1.0	1.0		ApUpCpGp
12		170	180				1.0	1.1			pApGp
13		135	275	260	√		1.0	2.0	1.9	√	m^1ApApApCpCpGp
14	290	150	160	160	√, √	1.9	1.0	1.0	1.0	√, √	$ApUpm^7GpDpm^5Cpm^5CpUpCpGp$
15[e]	280	85	175	420	√	3.2	1.0	2.0	4.9	√	$CpUpCpApUpt^6ApApCpCpCpUpGp$[e]

[a] The nucleoside analyses were performed as in the Materials and Methods section and Figure 3. [b] N refers to modified nucleosides that were detected (denoted by √) but not quantitated. [c] These peaks were not analyzed and the assignments are tentative. [d] Identified by unique sensitivity to limited venom phosphodiesterase digestion of intact $tRNA^{fMet}$ (J. Tropp, personal communication). [e] This peak corresponds to fragment 12.

termediate in which HSO_3^- adds across the 5,6 double bond. If the osmium were to form an adduct of cytidine and that adduct were to remain intact during the HSO_3^- treatment, then that cytidine should be protected from bisulfite catalyzed C → U conversion. Treatment of fragment 12′ with bisulfite, followed by Bio-Gel P-2 chromatography, demonstrated that bisulfite does not displace the osmium. Figure 4 compares the nucleosides produced by enzymatic digestion (which invariably releases the osmium) of fragments 12 and 12′ previously subjected to bisulfite treatment. The quantitation of the chromatograms (Table IV) shows that a single cytidine in fragment 12′ is protected from bisulfite conversion to uridine.

Osmium is Bound to the Pancreatic RNase Fragment t^6A-A-$C_{38}p$. To determine which cytidine was the site of modification, fragments 12 and 12′ were treated with pancreatic RNase and the digests analyzed by Pellionex chromatography (Figure 5). In comparing these digests the most striking result is the concomitant failure to recover both the fragment t^6A-A-C_{38}p and the osmium radioactivity. A similar result was observed with DEAE-cellulose chromatography. While the chromatographic phenomenon which underlies this extreme degree of retardation is not evident to us, these results strongly suggest that the osmium is bound to this oligonucleotide.

Osmium Interferes with Pancreatic RNase Cleavage of the $C_{38}pC_{39}$ Phosphodiester Bond. Comparison of the mononucleotides released from fragments 12 and 12′ by pancreatic RNase (Table V) revealed that digests of the osmated fragment were reproducibly deficient in a single 3′ CMP. These results are consistent with the osmium being attached to C_{38} since, as shown in the sequence included in Figure 5, the $Os(pyridine)_2$ moiety might be expected to sterically interfere with the cleavage of the $C_{38}pC_{39}$ phosphodiester bond, leaving the fragment t^6A-A-$^{Os}C_{38}$-Cp intact.

Osmium Protects C_{38} from Bisulfite Conversion to U. To more conclusively demonstrate that the osmium binding

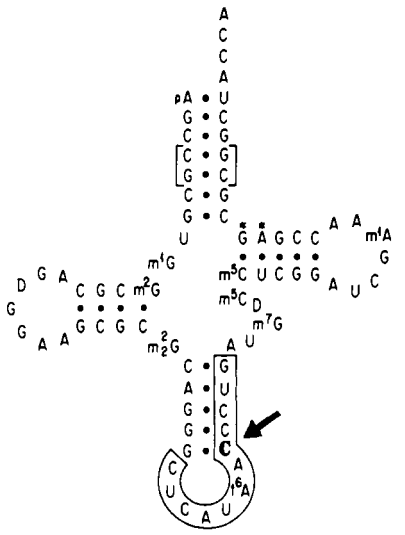

FIGURE 3: The cloverleaf representation of yeast tRNA^{fMet} (Simsek and RajBhandary, 1973). The box encloses fragment 12. Arrow points to C_{38} which is in bolder relief.

site is C_{38}, fragments 12 and 12′ were treated with bisulfite as previously described. The bisulfite treated oligonucleotides were then incubated with $NaIO_4$ to remove the osmium, and digested with pancreatic RNase. Figure 6 shows the elution profiles of the digest products on Pellionex. Whereas t^6A-A-U_{38}p is generated from fragment 12 due to the C → U conversion of C_{38}, the corresponding fragment from 12′ is t^6A-A-C_{38}p (Table VI). This demonstrates that the single cytidine protected by osmium is at position 38.

Os-tRNA^{fMet} is a Normal Substrate for Enzymatic Aminoacylation. Table I shows that 1.5 nmol of methionine are enzymatically accepted per A_{258} unit of Os-tRNA^{fMet} without detectable loss of covalently bound osmium. The time course of the charging reaction (Figure 7) is the same for both parent and Os-tRNA^{fMet} indicating that the productive interaction of yeast tRNA^{fMet} and yeast methionine:tRNA ligase is not disturbed. A similar result is observed using the *Escherichia coli* enzyme.

Discussion

The Mode of Attachment of Osmium to C_{38}. The original rationale for the preparation of the osmium derivative of yeast tRNA^{fMet} was to label the 3′-OH terminus with a bispyridyl osmate ester. The reactivity of Os(VI) toward cis diols was first shown by Criegee *et al.* (1942) and has been used by Behrman and coworkers (Subbaraman *et al.*, 1973) to prepare osmium derivative of nucleic acids. The ability to aminoacylate dissolved crystals of yeast Os-tRNA^{fMet} without releasing the osmium demonstrated that the osmium was not bound to the cis diol at the 3′ terminus (Schevitz *et al.*, 1972). The possibility that a reactive diol might exist due to the presence of a trialcohol amide in the supermodification of the *N*-(purin-6-ylcarbamoyl)threonine, t^6A (Kasai *et al.*, 1974), at position 36 was excluded by Dr. Kasai, in the laboratory of Dr. S. Nishimura, who kindly

TABLE IV: Composition of Bisulfite Treated Fragments 12 and 12′.

Sample	Relative Composition[a]			
	A	G	U	C
Fragment 12	2.0	1.0	3.2	4.9
Fragment 12 + bisulfite	1.9	1.0	8.0	<0.2
Fragment 12′	2.1	1.0	3.1	5.1
Fragment 12′ + bisulfite	2.0	1.0	7.0	1.1

[a] Nucleoside compositions relative to G. t^6A is not detected by the technique.

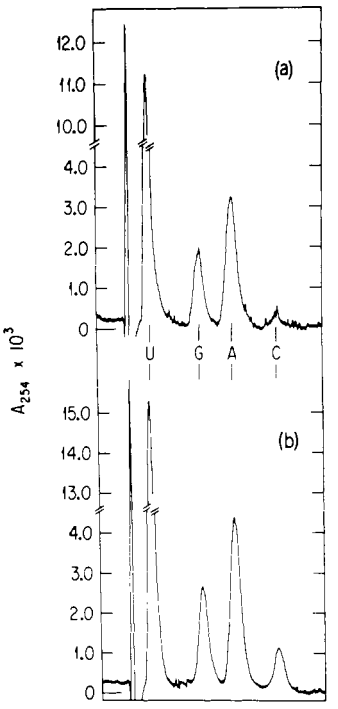

FIGURE 4: Nucleoside analysis of fragment 12 and fragment 12′ after treatment with bisulfite to convert cytidine to uridine. Approximately 1 nmol of oligonucleotide was dissolved in 0.5 ml of 2 M $NaHSO_3$-1 M Na_2SO_3 (pH 5.8), left at 20° for 24 hr, desalted on a 1 × 30 cm column of Bio-Gel P-2 in 0.02 M NH_4HCO_3 and lyophilized. The residue was treated with 0.5 ml of 0.4 M triethylammonium bicarbonate (pH 9.2) for 6 hr at 37° and lyophilized. The analysis of nucleoside composition was performed as described in Figure 3.

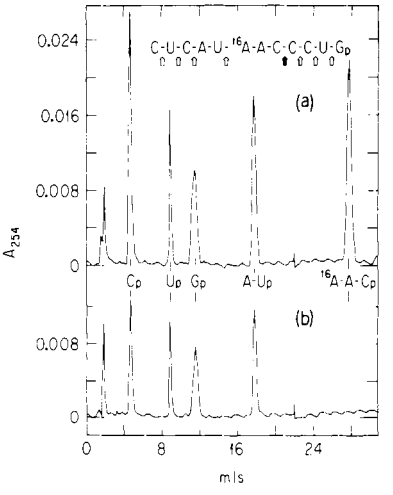

FIGURE 5: Pellionex chromatography of pancreatic RNase digests of fragment 12 (a) and fragment 12′ (b). Arrows indicate sites to be cleaved by pancreatic RNase. Shaded arrow indicates the phosphodiester linkage likely to be protected by extensive modification of C_{38}. Elution was carried as in Figure 1c.

TABLE V: Composition of Fragments from Pellionex Chromatography of Pancreatic RNase Digests of Fragments 12 and 12′.

				ApUp[b]				t⁶ApApCp[b]			
	Cp	Up	Gp[a]	U	G	A	C	U	G	A	C
Expected	4	2	1	1	0	1	0	0	0	1	1
Fragment 12	4.0	2.1	1.0	1.1	0.0	1.0	0.0	0.0	0.0	1.0	0.9
Fragment 12′	3.0	1.9	1.0	0.9	0.0	1.0	0.0		Not eluted		

[a] The mononucleotides released by pancreatic RNase digestion were quantitated directly from the chromatographic profile (Figure 5) and are given relative to Gp. [b] These fragments were collected and analyzed by nucleoside analysis as described in the Materials and Methods section and Figure 3. t⁶A is not detected by this analysis.

TABLE VI: Composition of Fragments from Pellionex Chromatography of Pancreatic RNase Digests of HSO_3^- and IO_4^- Treated Fragments 12 and 12′.

				ApUp[b]				t⁶ApApCp[b] (or t⁶ApApUp[b])			
	Cp	Up	Gp[a]	U	G	A	C	U	G	A	C
Fragment 12	0.0	5.8	1.0	0.9	0.0	1.0	0.0	0.9	0.0	1.0	0.0
Fragment 12′	0.0	6.1	1.0	1.1	0.0	1.0	0.0	0.0	0.0	1.0	1.0

[a] The mononucleotides released by pancreatic RNase were quantitated directly from the chromatographic profile (Figure 6) and are given relative to Gp. [b] The fragments were collected and analyzed by nucleoside analysis as described in the Materials and Methods section and Figure 3. t⁶A is not detected by this analysis.

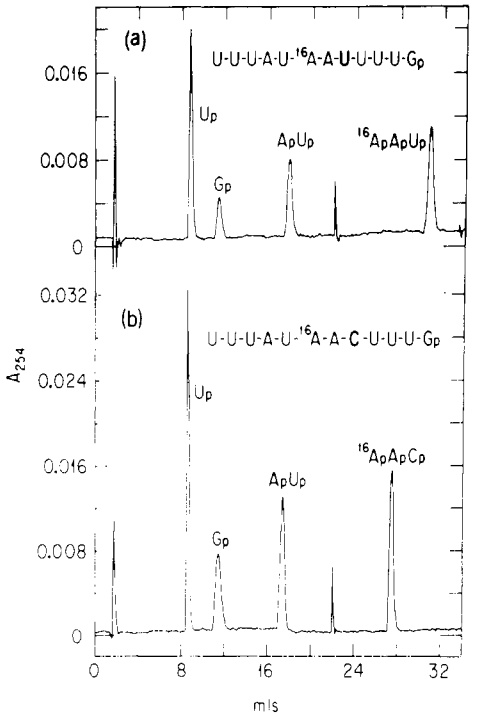

FIGURE 6: Pellionex chromatography of pancreatic RNase digests of fragment 12 (a) and fragment 12′ (b) having been treated successively with bisulfite to convert C to U and periodate to remove Os from the oligonucleotide. The elution conditions were as described in Figure 1c. The inserts show the putative sequences of the oligonucleotides following HSO_3^-/IO_4^- treatment.

verified that our samples of yeast $tRNA^{fMet}$ contained the usual t⁶A modification (personal communication). Osmium, as OsO_4, will readily add across the 5,6 double bond of a pyrimidine base to form a hydrolytically labile adduct which in the presence of pyridine forms a stable bispyridyl osmate ester (Beer *et al.*, 1966; Burton and Riley, 1966; Subbaraman *et al.*, 1971). We suggest that the crystals of yeast $tRNA^{fMet}$ react with $K_2OsO_2(OH)_4$ to form such an irreversible bispyridyl adduct of C_{38} and that the attacking reagent is not Os(VI) but a highly transient species of Os(VIII) produced in trace amounts by disproportionation of Os(VI) during the three weeks necessary to carry out the reaction (Subbaraman *et al.*, 1972). Figure 8 depicts a possible course for the reaction.

Stereochemical Requirements for a Stable Osmium Adduct. The specificity of this reaction is apparently dependent, in some unknown way, on the chemical and steric constraints unique to the crystal since in a solution (approximating the stabilizing mother liquor) $K_2OsO_2(OH)_4$ or OsO_4 in the presence of pyridine reacts with $tRNA^{fMet}$ at many sites. Moreover, the nature of the complex produced in the crystals was distinctive in that the osmated $tRNA^{fMet}$ isolated from the solution reactions appeared colorless or amber in solution, while dissolved crystals of Os-$tRNA^{fMet}$ or solutions of fragment 12′ appeared pale blue at concentrations exceeding $\sim 10^{-6}$ M.

The fact that the isolated T_1 fragment retains the unique blue complex suggests that whatever stereochemical factors specify the formation of the osmium adduct in the crystal, interactions with neighboring functional groups within the fragment are sufficient to stabilize it. Moreover, it appears that interactions with other residues in the T_1 fragment are *necessary* as well as sufficient for stability. This follows from the fact that total enzymatic digestion of fragment 12 to nucleosides completely disrupts the adduct. The osmium adduct apparently does not involve residues 39–42 of the anticodon stem. This follows from the observation that the

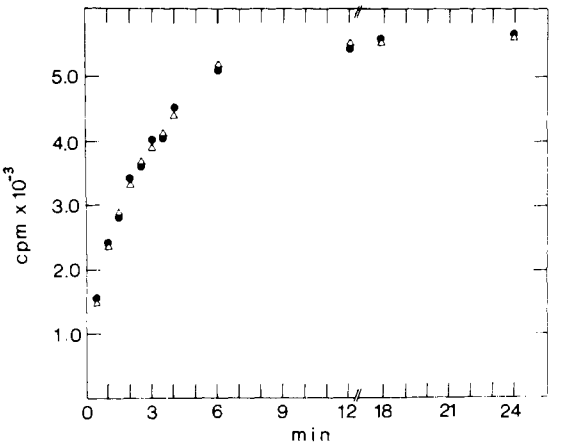

FIGURE 7: The time course of enzymatic aminoacylation of Os-tRNA^{fMet} (Δ) and tRNA^{fMet} (●) by yeast methionine:tRNA ligase. The reaction mixtures contained 0.010 M $MgCl_2$, 0.003 M ATP, 0.001 M dithiothreitol, 0.2 mg/ml of bovine serum albumin, 3.0×10^{-5} M methionine ([^{14}C]Met 50 Ci/mol; 5.10×10^4 cpm/nmol), 0.050 M Tris-HCl (pH 7.5), 0.40 A_{258} unit of tRNA^{fMet}/ml or 0.425 A_{258} unit of Os-tRNA^{fMet}/ml, and yeast enzymes. The reactions were incubated at 37° and 100 μl aliquots were removed periodically to determine the acid precipitable radioactivity according to Hoskinson and Khorana (1965). The concentrations of the tRNAs were adjusted to produce the same maximum levels of methionine incorporation.

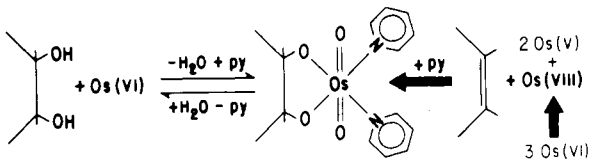

FIGURE 8: Schematic summary of pathways leading to osmium bispyridyl adduct (Subbaraman *et al.*; 1971,1972,1973). Bold arrows indicate pathway suggested here.

adduct is no more stable in the native molecule, where these residues presumably are constrained in a hydrogen-bonded helical stack (Kearns and Shulman, 1974; Kim *et al.*, 1973; Schevitz *et al.*, 1974), than it is in fragment 12, which has no sequence complement (Simsek and RajBhandary, 1972). We therefore suggest that the stability of the osmium bispyridyl adduct of C_{38} is enhanced by a functional group or groups from neighboring residues which are constrained in an appropriate three-dimensional arrangement by an intact segment of the anticodon loop. It is tempting to speculate that the oxygen functions of the threonyl moiety of the t^6A at position 36 form a ligand of the adduct. Preliminary model building studies of the anticodon stem and loop regions of yeast tRNA^{fMet} (Schevitz *et al.*, 1974) indicate that the formation of a bispyridyl osmate diester across the double bond of C_{38} is stereochemically compatible with the Watson–Crick base pairing (Kearns and Shulman, 1974) and acceptance of a threonyl oxygen function of the t^6A.

Aminoacyl Ligase Interaction with the Anticodon Loop. The normal aminoacylation of Os-tRNA^{fMet} by the methionine:tRNA ligases from both yeast and *E. coli* is of interest since Schulman and Goddard (1973) have shown that in the *E. coli* system bisulfite conversion of C → U in the anticodon causes rejection of the tRNA^{fMet} as a substrate. However, the ligase, whether from *E. coli* or baker's yeast, is apparently insensitive to substantial structural changes immediately to the 3′ side of the anticodon since it charges both *E. coli* and yeast tRNA^{fMet}, the former having an unmodified adenosine at the position corresponding to t^6A in the yeast tRNA^{fMet}. It is therefore not surprising that a substantial structural change involving the sequence, t^6A-A-C_{38}, does not affect the ligase interaction with yeast tRNA^{fMet}.

A Heavy Atom Marker for the Position of C_{38} in the Electron Density Map. This study has been carried out in parallel with the crystallographic structure determination of yeast tRNA^{fMet} (Schevitz *et al.*, 1974). Having established that the osmium is attached to C_{38}, the osmium atom becomes an invaluable aid in the interpretation of the low resolution electron-density map and complements the work of Pasek *et al.* (1973) who marked the position of C_{72} in the electron-density map by preparing a crystalline derivative in which C_{72} was enzymatically replaced by iodocytidine.

Although preliminary results indicate that there may be some significant differences between the three-dimensional structures of yeast tRNA^{fMet} (Schevitz *et al.*, 1974) and yeast tRNA^{Phe} (Kim *et al.*, 1973), there is a suggestion that the overall dimensions of the two molecular species are comparable since the distance between the heavy atom markers at C_{38} and C_{72} (54 Å) is in close agreement with the corresponding distance in the model of tRNA^{Phe} proposed by Kim *et al.*, (1973) (A. McPherson, G. Quigley, A. Rich, N. Seeman, and P. Sigler, unpublished observations).

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Ribonucleic Acid Ligase Activity of Deoxyribonucleic Acid Ligase from Phage T4 Infected *Escherichia coli*†

Hiroshi Sano and Günter Feix*

ABSTRACT: This study presents evidence that the DNA ligase of phage T4 purified according to the procedure of Weiss *et al.* (Weiss, B., Jacquemin-Sablon, A., Live, T. R., Fareed, G. C., and Richardson, C. C. (1968), *J. Biol. Chem. 243*, 4543) can be used as an RNA ligase. Working with high enzyme concentrations, with short oligoribonucleotides, and at 0°C, oligo(rU), oligo(rI), and oligo(rC) could be joined head-to-tail, respectively, in the presence of the corresponding complementary polyribonucleotides. Oligo(rA), however, in combination with poly(rU) or oligo(rU) could not be covalently linked under the conditions used.

Deoxyribonucleic acid ligase activities are implicated in replication, repair, and most probably also in recombination of double-stranded DNA in procaryotic and eukaryotic cells (Klein and Bonhoeffer, 1972; Radding, 1973). They catalyze the formation of phosphodiester bonds between the 5′-phosphoryl group and the 3′-hydroxyl group of nicks (interruptions of one strand) in double-stranded DNA structures (Richardson, 1969). Because of this activity, DNA ligases have become helpful tools in several synthetic reactions *in vitro* (Goulian *et al.*, 1967; Agarwal *et al.*, 1970; Lobban and Kaiser, 1973). Recently, it was found that the DNA ligase of phage T4, a polynucleotide ligase studied rather extensively, will also catalyze the end-to-end joining of bihelical DNA with fully base paired termini (Sagaramella and Khorana, 1972). In addition to this reaction, DNA ligase of phage T4 will also accept DNA-RNA hybrid structures as substrates, which has been demonstrated by the head-to-tail joining of various oligodeoxyribonucleotides on a polyribonucleotide template and *vice versa* (Kleppe *et al.*, 1970; Fareed *et al.*, 1971). Similar experiments, however, using oligo(rA)-poly(rU) or oligo(rU)-poly(rA)[1] as substrate combinations were unsuccessful (Kleppe *et al.*, 1970; Fareed *et al.*, 1971), and led to the conclusion that DNA ligase of phage T4 might not work with double-stranded RNA structures as substrate. During our attempts to connect RNA molecules covalently with each other, we restudied the DNA ligase of phage T4 and found that this enzyme can indeed be used as an RNA ligase if RNA-RNA structures other than oligo(rA)-poly(rU) are offered as substrates. It will be shown that under the conditions used (high enzyme concentration, rather short oligonucleotides, low temperature) DNA ligase of T4 does behave as an RNA ligase connecting oligoribonucleotides in various RNA-RNA combinations in a head-to-tail manner with the only exception that of oligo(rA)-poly(rU).

Experimental Section

Materials

DNA ligase was prepared principally according to the method described by Weiss *et al.* (1968) with a modification introduced by Knopf (1974). T4 am 4647 infected *Escherichia coli* strain B41 cells (50 g) (detailed procedure will be given elsewhere) were homogenized, treated by streptomycin, fractionated with ammonium sulfate, dialyzed, and loaded on a DEAE-cellulose column (4.5 × 11 cm) equilibrated with 10 mM Tris-HCl (pH 7.4)-1 mM β-mercaptoethanol (TM buffer), and eluted with 1000 ml of a linear gradient from 0 to 0.3 M sodium chloride in TM

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[1] The abbreviations used are specified in *Biochemistry 9*, 4022 (1970).