Photobinding of 8-Methoxypsoralen to Transfer RNA and 5-Fluorouracil-Enriched Transfer RNA†

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ABSTRACT: The photobinding of [^{3}H]8MOP to tRNA upon irradiation at 365 nm in the absence of O_2 was determined by gel filtration. The maximum photobinding was found to be ca. 4 mol of 8MOP per mol of tRNA and 5FU-tRNA, with an overall quantum yield of 2.3×10^{-3}. The photobinding kinetics for 8MOP-tRNA showed an apparent induction period or sigmoidal kinetic curve, indicating a specific initial photobinding site on tRNA which was identified as 4-thiouridine at position 8 from the 5′-end of *Escherichia coli* tRNA. Photobinding of 8MOP to 5FU-tRNA proceeded without an apparent induction period. 8MOP-tRNA and 8MOP-5FU-tRNA adducts were characterized by absorption, fluorescence, and CD spectroscopy. A modified procedure was also developed to analyze the nucleoside composition in modified 8MOP-tRNA and 8MOP-5FU-tRNA. The results showed that 8MOP photochemically added mainly to pyrimidine bases. The photobinding of 8MOP changed the conformation (secondary in particular) of tRNA and inhibited aminoacyl-tRNA synthetase activity.

Model studies of skin photosensitization and carcinogenesis of psoralens have centered on photomodifications of DNA by psoralens, while little work has been done on rRNA (Rodighiero et al., 1970), tRNA, and proteins. Mizuno and his coworkers (1974) qualitatively examined the effect of 8MOP and near-UV irradiation on the conformation and function of yeast tRNA.

tRNA exhibits a number of biochemical functions. In addition to its role in ribosome-directed translation processes, tRNA plays a role in cell wall synthesis, regulation of certain enzyme syntheses, amino acid transport and acts as primer for reverse transcriptase directed DNA synthesis. It is thus of interest to ascertain the role of psoralens in the photomodification of tRNA and its biochemical consequence. An additional motivation for the present study was to explore psoralens as an optical probe in elucidating various functions of tRNA (e.g., probe for interactions between aminoacyl-tRNA synthetase and tRNA).

The present report describes a detailed study of the interaction of photoexcited 8MOP[1] with tRNA and 5FU-tRNA isolated from *E. coli* B. The relatively small (tRNA) polynucleotide chain also serves as a useful model to characterize the binding mechanism of excited psoralens (furocoumarins) to nucleic acids and to correlate this with biological activity.

The advantages of using 5FU-tRNA for this investigation are the following: (1) 5FU-tRNA has the same biological activity in terms of aminoacylation as normal tRNA (Kaiser, 1969a; Johnson et al., 1969; Horowitz et al., 1974; Ofengand et al., 1974). (2) Previous studies in our laboratory indicate that 5FU is the most reactive base toward photoreaction with furocoumaryl compounds (Harter et al., 1974; McInturff, 1975). Thus, 5FU-tRNA is useful in preparing the psoralen-nucleic acid photoadducts for chemical analysis. (3) It is known that 5FU can replace uracil and a number of uracil-derived minor bases in RNA of bacterial (Horowitz and Chargaff, 1959), viral (Mandel, 1969), and higher organism sources (Heidelberger, 1965). Consequently, the complexity of photoreactions involving minor bases is effectively circumvented; in particular, the substitution of 4-thiouracil, a minor base in bacterial tRNA, with 5FU will eliminate many complications that are otherwise encountered in the spectroscopic study of photoadducts.

Experimental Section

Materials. 8-Methoxypsoralen (8MOP) and [^{3}H]8MOP were used as described in the preceding paper of this issue. Tritium-labeled potassium borohydride was Amersham-Searle No. TRK-293. Ribonuclease T_1 (grade III) and ribonuclease A (type 1A) were from Sigma Chemical Co. Snake venom phosphodiesterase (code VPH) and bacterial alkaline phosphatase (code BAPF) were from Worthington Biochemical Corp. Thin-layer cellulose sheets without fluorescent indicator from E. Merck Laboratory (5502) were used for nucleoside composition analysis and those from Eastman Kodak Co. were used for other analytical purposes. RP-R54 Royal X-Omat medical x-ray film was from Eastman Kodak Co. Unfractionated tRNA from *E. coli* B was purchased from Schwarz Bioresearch and further chromatographed on a Sephadex G-100 column using 1 M NaCl as the eluting solvent. *E. coli* $tRNA^{Phe}$ was from Boehringer Mannheim Biochemicals.

Bacterial Growth. E. coli strain B grown in nutrient agar was transferred to the glucose-salts medium of Demerec and Cahn (1953). If bacteria did not grow in this medium, 1% peptone was added to facilitate their growth. Mass growth of bacteria was performed in 15-L fermentors (New Brunswick Scientific Co.) at 37 °C. Addition of solid 5FU and thymine to a final concentration of 25 μg/mL each was made when the OD measurement at 650 nm was between 0.3 and 0.4 (corresponding to the early exponential phase of growth). Cells after 3 to 4 h of incubation were chilled and harvested with a continuous-flow centrifuge (Sorvall KSB-R), washed twice with 0.01 M Tris-HCl (pH 7.4)-0.01 M magnesium acetate buffer, and stored at −20 °C until used.

Preparation of 5-Fluorouracil-Enriched tRNA (5FU-tRNA). 5FU-tRNA was isolated as described by Horowitz et al. (1974). Following the rupture of 40 g of cells by a French

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[1] Abbreviations used: 5FU, 5-fluorouracil; 5FU-tRNA, 5-fluorouracil-enriched tRNA; 8MOP, 8-methoxypsoralen; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DEAE, diethylaminoethyl; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

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pressure cell at 10 000–12 000 psi, cell debris was removed by centrifugation at 20 000*g* for 20 min. Ribosomes were pelleted by ultracentrifugation at 105 000*g*. The ribosome-free supernatant (typically 135 mL) was extracted with phenol and sodium dodecyl sulfate to remove proteins (Johnson et al., 1969). tRNA was then deacylated by incubation at 37 °C in 1.8 M Tris-HCl buffer (pH 8.0) for 90 min (Sarin and Zamecnik, 1964). Contaminating RNA was separated from tRNA by chromatography on Sephadex G-100 using 1 M NaCl as eluting solvent. tRNA which contained little or no 5FU was completely eluted from the DEAE-cellulose column with 0.325 M NaCl in 0.02 M Tris-HCl buffer (pH 8.9). 5FU-tRNA was then eluted with an upward linear gradient of 0.325 to 0.6 M NaCl in 0.02 M Tris-HCl buffer (pH 8.9). Nucleoside analysis of 5FU-tRNA was carried out as described later.

Photobinding of 8-Methoxypsoralen to tRNA or 5FU-tRNA. Samples (typically 10 mL) containing equal molar concentrations of 8MOP (cold or ^{3}H-labeled) and tRNA (in moles of nucleotide; assuming average molecular weight of each nucleotide is 340) in either distilled H_2O or 0.01 M Tris-HCl (pH 7.4)–0.01 M magnesium acetate buffer were deoxygenated and irradiated at 365 nm as described in the preceding paper of this issue (Ou et al., 1978). After irradiation, the sample was chromatographed on Sephadex G-50 using 1 M NaCl as the eluting solvent. Fractions corresponding to tRNA were pooled and precipitated in 2 volumes of ethanol at −20 °C. The precipitates were then collected by centrifugation at 20 000*g* for 20 min. The contaminants were removed by extensive dialysis against either doubly distilled water or 0.01 M Tris-HCl (pH 7.4)–0.01 M magnesium acetate buffer. Samples containing tRNA or 8MOP alone served as references.

The binding ratio of 8MOP to tRNA was calculated from the radioactivity of purified 8MOP-bound tRNA. The extinction coefficient of tRNA used for the calculation was $\epsilon_{1cm}^{0.1\%}$ 23.9 at 260 nm for 5FU-tRNA and 23.3 for tRNA (Kaiser, 1969b).

Spectroscopic Characterization of Photoadducts. Purified 8MOP–tRNA and 8MOP–5FU-tRNA were used for spectroscopic measurements. The absorption, CD, and fluorescence spectra of all photoadducts were recorded as described in the preceding paper of this issue (Ou et al., 1978).

Preparation of $NaBH_4$-Reduced Irradiated tRNA or 8MOP–tRNA. Reduction of the irradiated control tRNA and 8MOP–tRNA to a highly fluorescent derivative was carried out by a modified method of Krauskopf et al. (1972) as described by Ofengand and Bierbaum (1973).

Preparation of Aminoacyl-tRNA Synthetases. Aminoacyl-tRNA synthetases free of tRNA were prepared at 4 °C by a slight modification of the method as described by Kelmers et al. (1965). *E. coli* B cells (15 g) were dispersed in 60 mL of buffer (0.01 M Tris-HCl, pH 7.4, containing 0.01 M magnesium acetate and 0.001 M glutathione) and disrupted by passage through a French pressure cell at 10 000 to 12 000 psi. The broken cell preparation was diluted with 4 volumes of buffer and centrifuged at 35 000*g* for 40 min and then at 100 000*g* for 3 h to remove particulate matter. The supernatant was collected and nucleic acids were then precipitated by the slow addition of 0.1 volume of 10% streptomycin sulfate (freshly prepared with cold deionized H_2O). After stirring for 3 h, the precipitate was removed by centrifugation at 16 000*g* for 10 min. The supernatant solution was maintained at pH 7.5 during the addition of solid ammonium sulfate at 65% saturation. After stirring for 0.5 h, the precipitate was collected by centrifugation at 16 000*g* for 10 min. The residues were redissolved in 40 mL of buffer and dialyzed overnight against 8 L of buffer. Finally, 0.25 volume of glycerol was added to the enzyme solution and stored at −60 °C. The protein concentration estimated by A_{280}/A_{260} ratio was about 2 mg/mL. The contaminating nucleic acid was only 0.4%.

Amino Acid Acceptor Activity. Aminoacylation of tRNAs and their photoadducts was carried out as described by Kelmers et al. (1965) or Pestka (1966) and modified by Johnson et al. (1969). A 0.5-mL reaction mixture contained 50 μmol of magnesium acetate, 1 μmol of ATP, 2.5 μmol of KCl, 1.5 nmol of a ^{14}C-labeled L-amino acid, 3 nmol of each of the other 19 amino acids, 0.5 μmol of CTP, 0.02 to 2.0 A_{260} units of tRNA, and sufficient crude aminoacyl-tRNA. After incubation at 37 °C for 30 min, the reaction was stopped by the addition of 7% cold trichloroacetic acid; 0.5 mg of bovine serum albumin was added as carrier. The precipitate was collected on Millipore filters, dried, and counted in a Beckman liquid scintillation counter in a PPO–POPOP–Triton X-114–xylene cocktail.

Chemical Analysis of 8MOP–tRNA and 8MOP–5FU-tRNA. [^{3}H]8MOP-tRNA (or [^{3}H]8MOP–5FU-tRNA) was completely digested into nucleosides plus [^{3}H]8MOP-nucleoside (including nucleoside-[^{3}H]8MOP–nucleoside, a cross-linking product) by an enzyme mixture containing ribonuclease A, ribonuclease T_1, snake venom phosphodiesterase, and alkaline phosphatase under the conditions used for nucleoside composition analysis as described later. The products were separated by TLC on cellulose and visualized on x-ray film by low-temperature fluorography. The solvent systems used for TLC were: (a) acetonitrile–ethyl acetate–1-butanol–2-propanol–6 N ammonia (7:2:1:1:2.7, v/v), (b) *tert*-amyl alcohol–methyl ethyl ketone–acetonitrile–water–formic acid (88%) (4:2:1.5:2:1.5:0.18, v/v), (c) 1-propanol–water (3:7, v/v), and (d) 1-propanol–water (7:3, v/v).

Nucleoside Composition Analyses of 8MOP–tRNA and 8MOP–5FU-tRNA. The nucleoside composition of 8MOP–tRNA and 8MOP–5FU-tRNA was analyzed by the chemical tritium-labeling method developed by Randerath et al. (1972, 1974) and modified in our laboratory, as detailed in Ou (1977).

Results

Chromatography and Base Composition Analysis of 5FU-tRNA. After deacylation, tRNA was precipitated in ethanol and collected by centrifugation. The precipitates were then redissolved in 25 mL of 1 M NaCl and passed through a Sephadex G-100 column using 1 M NaCl as the eluting solvent; the contaminating RNA and phenol were separated from tRNA. The major peak contains tRNA, low 5FU-substituted tRNA, and 5FU-tRNA. Since 5FU has a lower pK_a than uracil (Heidelberger, 1965), 5FU-tRNA has a greater net negative charge than tRNA or low 5FU-substituted tRNA at certain pH values. When tRNA from Sephadex G-100 was applied to a DEAE-cellulose column and washed with 0.325 M NaCl in Tris-HCl buffer (pH 8.9), tRNA and low 5FU-substituted tRNA were eluted out completely. 5FU-tRNA was eluted with an upward linear gradient of a 0.325 to 0.6 M NaCl in Tris-HCl buffer (pH 8.9) as shown in Figure 1. Nucleoside composition analysis of 5FU-tRNA from DEAE-cellulose chromatography by the chemical tritium-labeling method showed that more than 90% of its uridine residues or derivatives (such as pseudouridine, dihydrouridine, and ribothymidine) was replaced by 5FU. The major nucleoside composition of 5FU-tRNA remains essentially unchanged except for a small replacement of cytidine by 5-fluorocytidine (Horowitz et al., 1974). 4-Thiouridine content in 5FU-tRNA was determined

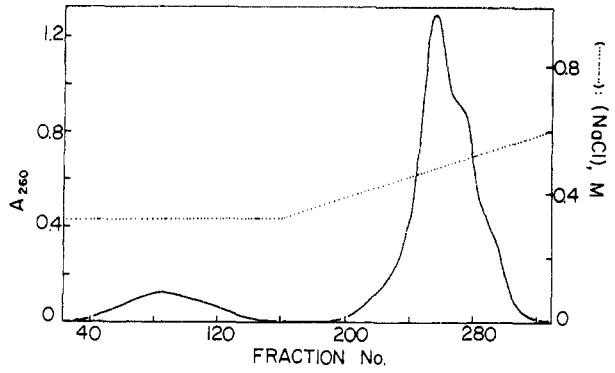

FIGURE 1: DEAE-cellulose column chromatography of a mixture of 5FU-enriched tRNA and tRNA. Mixed tRNA (60 mg) obtained from Sephadex G-100 chromatography was applied to a DEAE-cellulose column (2.5 × 38 cm) and 14.2-mL fractions were collected. tRNA which contained little or no 5FU was eluted from the column with 0.325 M NaCl in 0.02 M Tris-HCl buffer (pH 8.9). 5FU-enriched tRNA was then eluted with an upward linear gradient of 0.325 to 0.6 M NaCl in 0.02 M Tris-HCl buffer (pH 8.9). The absorbance of each fraction was determined. Aliquots of fraction 230 to 320 were pooled and precipitated in ethanol.

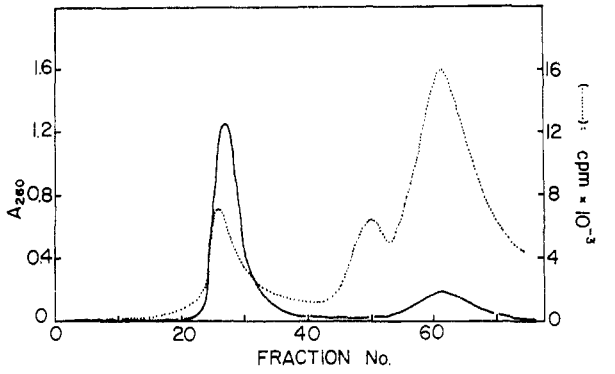

FIGURE 2: Sephadex G-50 column chromatography of the irradiated solution containing equimolar concentrations of 5FU-tRNA and [^{3}H]-8MOP. Irradiation was carried out as described under Experimental Section. Irradiation time was 12 h at 365 nm (6.5×10^{14} quanta s^{-1} 10 mL^{-1}). The concentration of 5FU-tRNA (in moles of nucleotide) or 8MOP was 2.27×10^2 μM. The eluting solvent was 1 M NaCl. The flow rate was 0.5 mL/min and 3-mL fractions were collected and assayed both for radioactivity (· · ·) and for absorbance at 260 nm (—).

by the absorbance ratio A_{337}/A_{260} as described by Doctor et al. (1969). There was only 10% of the normal amount of 4-thiouridine present in the 5FU-tRNA.

8MOP-tRNA and 8MOP-5FU-tRNA Photoadducts. A reaction mixture (10 mL) containing equimolar concentrations of [^{3}H]8MOP and tRNA (or 5FU-tRNA) was chromatographed on a Sephadex G-50 column after irradiation at 365 nm. The elution profile is shown in Figure 2. The radioactivity associated with tRNA or 5FU-tRNA indicated covalent binding of 8MOP to tRNA, since control samples containing tRNA and [^{3}H]8MOP without irradiation did not give any radioactivity at the tRNA peak. Elution profiles of the reaction mixture obtained from irradiation of a sample containing tRNA and 8MOP in the presence of O_2 showed that tRNA was fragmented by singlet oxygen generated by the triplet 8MOP.

Figure 3 shows the photoinduced binding of 8MOP-tRNA. Photobinding of 8MOP to 5FU-tRNA was much faster than to tRNA during the initial stage due to the high reactivity of 5FU. The maximum incorporation of 8MOP into both normal tRNA and 5FU-tRNA was about the same at 16 h of irradiation time (~3.5 mol of 8MOP/mol of tRNA), but tRNA showed a sigmoidal binding curve. The overall quantum yield

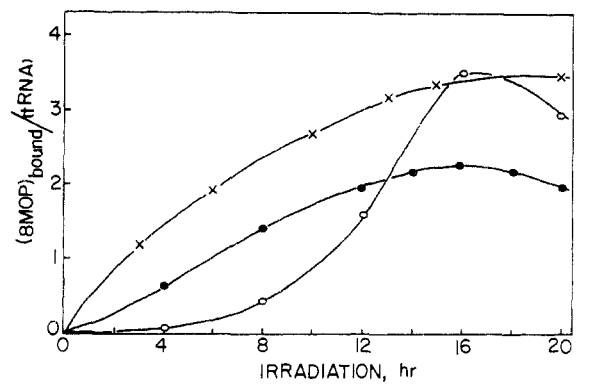

FIGURE 3: Photobinding kinetics of [^{3}H[8MOP to 5FU-tRNA (x-x), native tRNA (O-O), and preirradiated tRNA (●-●).

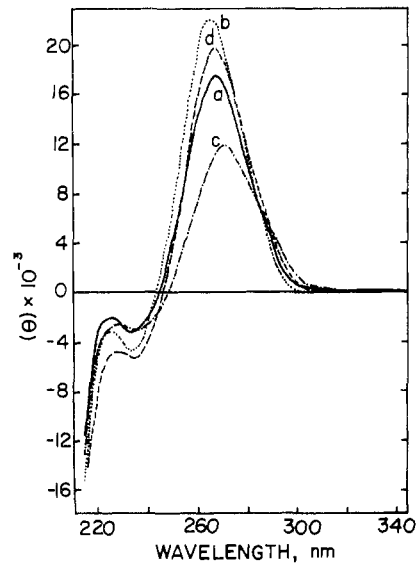

FIGURE 4: (a) CD spectra of 8MOP-tRNA in water; (b) control tRNA; (c) 8MOP-5FU-tRNA; and (d) control 5FU-tRNA.

of the formation of 8MOP-tRNA and 8MOP-5FU-tRNA adducts at 16 h of irradiation time was 2.3×10^{-3} (~0.07 after correction for free 8MOP initially present). When tRNA was preirradiated at 365 nm for 12 h prior to the addition of 8MOP, the binding ratio was lower than the native tRNA binding ratio. Furthermore, the binding curve was no longer sigmoidal. Prolonged irradiation for more than 16 h caused some dissociation of 8MOP from tRNA.[2]

Absorption spectra of 8MOP-tRNA and 8MOP-5FU-tRNA showed a slight blue shift (1 to 2 nm) of the major band (258 nm) and a new abosrbance band at longer wavelengths (320 to 500 nm) as compared to the control sample. CD spectra of 8MOP-tRNA and 8MOP-5FU-tRNA showed a 3- to 4-nm red shift of the positive band; rotational strengths of both positive and negative bands are much less than the control tRNA samples (Figure 4).

8MOP-5FU-tRNA showed a distinctly different fluorescence spectrum ($\lambda_{F,max}$ ~488 nm) from either the spectrum of control 5FU-tRNA or 8MOP itself. The control 5FU-tRNA showed no fluorescence. The 8MOP-tRNA showed heterogeneous emission (i.e., two or more adduct species). The control tRNA showed an emission maximum at 400 nm when excited at 330 nm. 8MOP-tRNA showed two emission maxima,

[2] This may also be attributed to the loss of ^{3}H via exchange with unmodified nucleotides (e.g., Sims et al., 1974; Meehan et al., 1976).

$\lambda_{F,max}$, at 400 and 480 nm. The corrected excitation and fluorescence spectra of $NaBH_4$-reduced samples of control tRNA and 8MOP-tRNA adduct showed excitation, λ_{max}, at 270 and 386 nm and a shoulder at 401 nm with emission, $\lambda_{F,max}$, at 445 nm. The 8MOP-tRNA adduct, however, showed a higher emission at longer wavelengths (>493 nm) presumably due to the emission of 8MOP-nucleotide adducts. These data confirm that the 4-thiouridine-cytidine adduct was one of the predominant emitting species. Comparing the corrected fluorescence intensity of both $NaBH_4$-reduced samples of control tRNA and 8MOP-tRNA and correcting for the overlapping intensity at 445 nm contributed by 8MOP-nucleotide adducts, we estimate that there is about 54% 4-thiouridine-cytidine adduct formed in 8MOP-tRNA. The fluorescence lifetime was 1.85 ns for 8MOP-5FU-tRNA and 1.0-2.5 ns for 8MOP-tRNA (Table I).

Chemical Analysis of [^{3}H]8MOP-5FU-tRNA. Among 20 different solvent systems tried, the mixture of 1-propanol and water (solvents C and D) gave the best separation of the products of the enzymatic digestion of the [^{3}H]8MOP-5FU-tRNA. Two major (R_f 0.23, 0.48 in solvent D) and six minor (R_f 0.27, 0.31, 0.35, 0.44, 0.58, 0.73) products were separated by two-dimensional chromatography using solvent C in the first dimension and solvent D in the second. These photoproducts were not photodissociated into component base and 8MOP with ultraviolet light (3.5-h irradiation at 255 nm) or with photosensitizer 2-anthraquinonesulfonate (3-h irradiation at 365 nm).

Nucleoside Composition Analysis of 8MOP-tRNA and 8MOP-5FU-tRNA. The standard method for the base composition analysis of photomodified tRNA as described by Randerath et al. (1972) yielded incomplete enzymatic digestion. An endonuclease, RNase T_1, specific for guanine residues was employed for the digestion of photomodified tRNA. The base composition of nonirradiated tRNA was the same as that of the irradiated tRNA digested by the enzyme mixture containing RNase T_1. However, the base composition of the irradiated tRNA and 5FU-tRNA digested in the absence of RNase T_1 was not satisfactorily determined due to incomplete digestion of tRNA and 5FU-tRNA.[3] Results on 8MOP-tRNA and 8MOP-5FU-tRNA digested in the presence of RNase T_1 are presented in Table II.[4] The accuracy of this method was tested on purified $tRNA^{Phe}$ (*E. coli*) with a known sequence. Table III summarizes the results. The agreement is excellent, except for the cytidine content which is often slightly overestimated in the chemical-labeling method (Randerath et al., 1972). The base contents of two minor nucleosides, 2-methylthio-N^6-isopentenyladenosine and 3-(3-amino-3-carboxypropyl)uridine, were not determined in this test because the locations of these two components were unknown. These results suggest that the method used is accurate and that the enzymes employed are capable of giving complete digestion of photomodified tRNA.[4]

[3] A complete set of the composition analysis data (including minor bases) is available in Ou (1977) and from the authors upon request.

[4] After two-dimensional separation by solvents A and B, the fluorogram showed that all the radioactivity remained at the origin. Therefore, the nucleoside which photoreacted with 8MOP and formed an 8MOP-nucleoside adduct would not migrate to the same position as the free nucleoside would. This would cause a decrease in the total content if there were photoaddition between this fraction of the base and 8MOP. In order to obtain the true base composition of 8MOP-tRNA and 8MOP-5FU-tRNA photoadducts for comparison with the control tRNA and 5FU-tRNA, respectively, a correction was made for the mol % of each base of 8MOP-tRNA and 8MOP-5FU-tRNA adducts by multiplying each mol % by a factor of 72/76. This factor was determined by the binding ratio of [^{3}H]8MOP and tRNA.

TABLE I: Fluorescence Lifetimes (in ns) of 8MOP-5FU-tRNA and 8MOP-tRNA.

Excitation wavelength (nm)	8MOP-5FU-tRNA		8MOP-tRNA	
	10 MHz[a]	30 MHz[a]	10 MHz[a]	30 MHz[a]
330	1.840	1.675		
338			1.430	2.530
350	1.812	1.908		
375	1.858	1.486	1.036	0.984

[a] All lifetimes reported here are measured by phase delay at two modulation frequencies indicated and at 25 °C (average of 20 to ~25 measurements).

TABLE II: Nucleoside Compositions of 8MOP-tRNA and 8MOP-5FU-tRNA and Control tRNA as Determined by the Chemical Tritium-Labeling Method. See Footnote 3 for Complete Data Including Minor Bases.

Nucleoside	mol % ± SD		Control tRNA or 5FU-tRNA[a]
	−RNase T_1	+RNase T_1[a]	
	8MOP-tRNA		
G	20.59	30.53 ± 0.25	30.81 ± 0.10
C	34.10	28.10 ± 0.16	29.81 ± 0.21
A	21.33	16.90 ± 0.10	18.55 ± 0.11
U	16.32	13.30 ± 0.12	14.20 ± 0.08
	8MOP-5FU-tRNA		
G	16.67	30.30 ± 0.24	29.80 ± 0.10
C	37.68	28.31 ± 0.33	28.24 ± 0.12
A	21.35	17.82 ± 0.14	19.17 ± 0.23
U	2.41	1.68 ± 0.02	1.46 ± 0.01
5FU	18.16	14.60 ± 0.23	18.36 ± 0.30

[a] Corrected for the content of 8MOP-nucleoside adduct as described under Results.

As shown in Table II, the 5FU content in 8MOP-5FU-tRNA was drastically decreased, indicating that 5FU was the most reactive site toward photoreaction with 8MOP.[3] In 8MOP-5FU-tRNA, there were 3.76 mol % of 5FU and 1.57 mol % of adenosine, equivalent, respectively, to 3 molecules of 5FU and 1.2 molecules of adenosine per molecule of 5FU-tRNA (assuming, on the average, 76 nucleotides per tRNA molecule) which reacted with excited 8MOP. Table II also shows the base composition of 8MOP-tRNA photoadduct.[3] There was a decrease in the content of adenosine, cytidine, and uridine, indicating that the 8MOP photoaddition occurred at these bases. From the differences between the base composition of 8MOP-tRNA and control tRNA, we obtained 1.3 molecules of cytidine, 1.25 molecules of adenosine, and 0.7 molecules of uridine per tRNA photoreacted with 8MOP.

Amino Acid Accepting Capacity. The amino acid accepting capacity of 8MOP-tRNA and 8MOP-5FU-tRNA decreased drastically in comparison with controls (irradiated tRNA without 8MOP and with nonirradiated tRNA in the presence of 8MOP). For example the Phe and Leu accepting capacity was 15.3 and 29 pmol/A_{260} unit, respectively, for 8MOP-tRNA and 15.4 and 16.2, respectively, for 8MOP-5FU-tRNA. Controls showed >75 pmol/A_{260}.

Discussion

Photobinding Kinetics. As was emphasized in the preceding paper of this issue (Ou et al., 1978), it is important to carry out irradiation of tRNA and 5FU-tRNA in the absence of O_2.

TABLE III: Nucleoside Compositions of *E. coli* $tRNA^{Phe}$, Control $tRNA^{Phe}$, and 8MOP-$tRNA^{Phe}$ as Determined by the Chemical Tritium-Labeling Method[a] (Standard Deviations Are Indicated).

Nucleoside	$tRNA^{Phe}$		Control $tRNA^{Phe}$		8MOP-$tRNA^{Phe}$			Expected,[d] In 74[b]
	mol %	In 74[b]	mol %	In 74[b]	mol %	In 74[b]	Corrected value[c]	
G	30.94	22.90 ± 015	30.96	22.91 ± 0.18	29.99	22.19	21.02 ± 0.24	23
C	28.95	21.42 ± 0.18	29.21	21.61 ± 0.21	28.54	21.12	20.01 ± 0.25	21
A	19.06	14.10 ± 0.13	18.52	13.71 ± 0.24	19.79	14.64	13.87 ± 0.11	14
U	12.17	9.00 ± 0.10	12.01	8.89 ± 0.13	12.15	8.99	8.51 ± 0.17	8 + 1[e]
ψ	3.40	2.52 ± 0.02	4.00	2.96 ± 0.04	4.34	3.21	3.04 ± 0.02	3
hU	2.79	2.06 ± 0.03	2.93	2.16 ± 0.03	2.92	2.16	2.05 ± 0.03	2
m^5U	1.42	1.05 ± 0.01	1.46	1.08 ± 0.01	1.46	1.08	1.02 ± 0.01	1
m^7G[f]	1.30	0.96 ± 0.03	0.92	0.68 ± 0.05	0.83	0.61	0.58 ± 0.02	1

[a] With RNase T_1 treatment. [b] Chain length = 76 nucleotides; 2-methylthio-N^6-isopentenyladenosine and 3-(3-amino-3-carboxypropyl)-uridine were subtracted; see text. [c] Corrected for the content of 8MOP-nucleoside; see text. [d] Barrell and Clark (1974). [e] 4-Thiouridine is recovered mainly as [^{3}H]uracil; see Randerath et al. (1972). [f] Corrected for 64% recovery.

Extensive chain breaking occurs as a result of singlet oxygen which is produced from the triplet sensitization of 8MOP.

The sigmoidal photobinding kinetics of 8MOP to tRNA (Figure 3) suggests a specific initial binding which results in significant conformation changes such as the secondary structural change monitored by CD (Figure 4). This behavior is absent in the photobinding of 8MOP to 5FU-tRNA, most likely reflecting the high reactivity of the 5FU base in the tRNA. Interestingly, the near-UV preirradiated tRNA shows no "induction" period, indicating that the specific binding site is no longer available in the preirradiated tRNA. The most likely specific site involved is 4-thiouridine at position 8, which becomes cross-linked to cytidine at position 13 upon near-UV preirradiation. This is further supported by the fact that 5FU-tRNA and yeast $tRNA^{Phe}$, both lacking 4-thiouridine, do not show the induction period and that the maximum photobinding of 8MOP to the near-UV preirradiated tRNA is reduced by approximately one 8MOP. It is well known that near-UV irradiation induces photocross-linking between 4-thiouridine and cytidine in *E. coli* tRNA in vitro and in vivo (Ofengand and Bierbaum, 1973; Favre et al., 1971; Carre et al., 1974; Ramabhadran et al., 1976). The preirradiation is also accompanied by secondary structural changes as monitored by CD and NMR (Bolton et al., 1977), thus providing a plausible explanation for the lack of induction period and apparent sigmoidal binding curve with the preirradiated tRNA.

Specific binding sites on tRNA and 5FU-tRNA are likely to be determined by two factors: (1) ground-state complex formation between 8MOP and tRNA which provides two or three possible intercalating base plates (see Kim, 1977), and (2) reaction between excited 8MOP and exposed bases (e.g., Schulman and Pelka, 1976). Both factors are probably involved in the photobinding of 8MOP to tRNA and 5FU-tRNA. In order to specifically assess the ground-state complexation, various spectroscopic methods (CD, absorption, and fluorescence) were attempted without success. However, the fluorescence polarization (P) in solution at room temperature was found to be the most sensitive method to detect the ground-state complex: $P = 0.022$ for free 8MOP with λ_{ex} 365 nm, $P = 0.1$ for 8MOP + 5FU-tRNA (1:15 P_i mixture); the latter value represents binding of about 1 8MOP per 30 nucleotides. From this result, the ground-state complex formation is seen to be the critical factor in determining the site and number of 8MOP photobinding to tRNA.

Photobinding Sites. The corrected excitation and fluorescence spectra of a $NaBH_4$-reduced sample of 8MOP-tRNA confirm that 4-thiouridine-cytidine dimer (54%) is one of the predominant emitting species, in addition to 8MOP-nucleoside adducts. Thus, the induction period and sigmoidal kinetics can be at least partly attributed to the photoreactions of 8MOP with 4-thiouridine (or cytidine) and 4-thiouridine with cytidine, both of which lead to similar conformation changes and enhanced photoreactivity of two to three additional tRNA bases toward 8MOP.

As to the photobinding sites, the chemical tritium-labeling method reveals adenosine, cytidine, and uridine (including 4-thiouridine which is determined as U in this method) to be the major photoreactive sites in 8MOP-tRNA (Table II). In the case of 8MOP-5FU-tRNA, 5FU is the most reactive site (Table II). Again, loss of approximately 1 adenosine is implicated. It should also be pointed out that all 8MOP-nucleoside adducts remained at the origin of the chromatogram, thus reducing the nucleoside content in terms of the mol % as determined by the modified chemical tritium-labeling method. Minor photoadducts in 8MOP-5FU-tRNA detected on TLC may well represent products involving residual minor pyrimidine bases,[3] isomers, and cross-linked dinucleoside adducts. The chemical tritium-labeling method cannot delineate these minor products.

From the chemical tritium-labeling results, we conclude that more than 70% of the bases involved in the photobinding with excited 8MOP are 5FU. The two major products resolved on TLC are probably 3,4- (weakly fluorescent) and 4′,5′-monadducts (strongly fluorescent), accounting for the apparent homogeneity of the fluorescence lifetime of 8MOP-5FU-tRNA. The absorption band at 320–400 nm is most likely due to the 4′,5′-monoadduct, since 3,4-dihydropsoralen and 3,4,4′,5′-tetrahydropsoralen do not absorb significantly in this region compared to 4′,5′-dihydropsoralen (Ou et al., 1978). The product distribution is far more complex for the 8MOP-tRNA photoadduct, as expected from the chemical tritium-labeling data (Table II), TLC results, and heterogeneous fluorescence lifetimes.

Conformational Changes and Amino Acid Accepting Capacity. In contrast to the results of Mizuno et al. (1974) and Kittler and Zimmer (1976), there is a marked change in the CD spectra of tRNA and 5FU-tRNA upon photobinding with 8MOP (Figure 4). The CD change involves a red shift of the positive CD band in the photoadducts and a decrease in the ellipticity, suggestive of the disruption of base stacking and helicity of the tRNA arms (Hashizume and Imahori, 1967). These changes are likely to affect the tRNA activity, as discussed below.

The loss of the amino acid accepting capacity of 8MOP-tRNA and 8MOP-5FU-tRNA can be accounted for by the

following possibilities: (1) disruption of secondary and/or tertiary structural changes (cf., CD results), (2) photobinding of 8MOP at the 3′-C-C-A arm (amino acid arm) and/or the anticodon arm, and (3) photobinding of 8MOP at or near the binding site on tRNA for aminoacyl-tRNA synthetase. The last mode of inhibition is most attractive in that the initial specific binding of 8MOP at 4-thiouridine could destroy the optimal contact with the binding site on the enzyme, since the inhibition of aminoacyl-tRNA synthetase is markedly reduced by the initial binding of 8MOP to tRNA and 5FU-tRNA. We are now exploring the possible use of 8MOP as an extrinsic optical probe for the interaction of tRNA with aminoacyl-tRNA synthetase and ribosomal binding site. Photocross-linking and isotope-labeling studies of tRNA–aminoacyl-tRNA synthetase complexes have shown that the enzyme binds along and around the interior of the L-shaped tRNA, with interactions at six sites extending from the 3′-terminus at one end of the L-shaped molecule to the anticodon loop located about 75 Å away at the other end (Schoemaker and Schimmel, 1974, 1976; Budzik et al., 1975; Schoemaker et al., 1975; Quigley and Rich, 1976).

Conclusion

The specific binding of up to four 8MOP per tRNA and 5FU-tRNA occurs, accompanied by significant conformational changes and the loss of amino acid accepting capacity of the photomodified tRNAs. Since tRNA participates in several functions other than its role in protein biosynthesis, photobiological activity of 8MOP is likely to involve contributions from the photoinduced interaction between 8MOP and tRNA.

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