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THE METHYLENETETRAHYDROFOLATE-MEDIATED BIOSYNTHESIS OF RIBOTHYMIDINE IN THE TRANSFER-RNA OF *STREPTOCOCCUS FAECALIS*: INCORPORATION OF HYDROGEN FROM SOLVENT INTO THE METHYL MOIETY

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SUMMARY

The methyl carbon of ribothymidine in the tRNA of *Streptococcus faecalis* is derived from 5,10-methylenetetrahydrofolate, not *S*-adenosylmethionine. Isotope labeling experiments have shown that the reduction of the methylene carbon of the folate cofactor to the methyl carbon of the modified residue involves a mechanism in which hydrogen from solvent is incorporated into the methyl moiety. Although the identity of the reducing agent involved directly in this novel methylation remains to be established, data suggest that reduced flavin serves this function *in vitro*.

INTRODUCTION

The biosynthesis of ribothymidine in loop IV of the tRNA of *Streptococcus faecalis* occurs at the polynucleotide level and involves a reaction in which 5,10-methylenetetrahydrofolate, rather than *S*-adenosylmethionine, serves as the one-carbon donor (1). A similar reaction appears to occur in *Bacillus subtilis* (1-4), *Bacillus cereus* (1) and some other (5) -- but not all (1) -- Gram-positive micro-organisms.

This reaction represents a previously unrecognized metabolic role for folate and the first example of a methylation of nucleic acid *not* involving *S*-adenosylmethionine.

The reaction is also of interest in view of the analogy it bears to the methylenetetrahydrofolate-dependent methylation of deoxyuridylate by thymidylate synthetase (6). However, in contrast to deoxythymidylate biosynthesis, in which 5,10-methylenetetrahydrofolate donates both the one-carbon unit (as $-CH_2-$) and the two electrons and hydrogen (from position 6 of the pteridine

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ring) necessary for the reaction, our data suggested that ribothymidine formation involves a different mechanism for the reduction of the methylene carbon (1). Specifically, we observed no incorporation of label into ribothymidine from [6-^{3}H]tetrahydrofolate either *in vitro* or *in vivo* and found a requirement for reduced flavin for ribothymidine biosynthesis *in vitro* (1).

We have purified to homogeneity the enzyme that catalyzes ribothymidine formation in *S. faecalis* tRNA in an effort to elucidate the mechanism of this novel reaction and its relationship to that catalyzed by thymidylate synthetase

MATERIALS AND METHODS

Reagent grade chemicals were generally used. (*dℓ*)-L-[6-^{3}H]Tetrahydrofolate (1.2 Ci/mmol), a gift of Dr. D.V. Santi, University of California, San Francisco, and (*dℓ*)-L-[6,7-^{3}H]tetrahydrofolate (10.5 mCi/mmol), prepared by reduction of folic acid with KB^3H_4 (Amersham) essentially as described (7), were used without dilution with unlabeled tetrahydrofolate. [^{14}C]Formaldehyde (44 mCi/mmol) was purchased from Amersham, ^3H_2O (5 Ci/ml) from ICN Pharmaceutical, ^2H_2O (99.8 mol % deuterium) from BioRad Laboratories, and bis-trimethylsilyltrifluoroacetamide (BSTFA) from Pierce Chemical. Dr. Santi also supplied purified *Lactobacillus casei* thymidylate synthetase.

Unless stated otherwise, experimental procedures were the same as those described previously (1). Reaction conditions were as follows: 40 mM Na-Bicine, pH 9.5, 40 mM Tris-HCl, pH 7.5, 180 mM NH_4Cl, 5 mM Na-EDTA, ≃100 mM 2-mercaptoethanol, 4 mM NADPH, 10 mM NADH, 0.25 mM FAD, ≃4 mM (*dℓ*)-L-5,10-methylenetetrahydrofolate, 10-80 μM tRNA(UΨC), enzyme; 0.100 ml, 37°C, N_2 atmosphere. Labeled compounds were used in specific experiments. For example, tRNA was methylated with methylene-[6-^{3}H]tetrahydrofolate, prepared with unlabeled formaldehyde (8), or [^{14}C]methylene-[6-^{3}H]tetrahydrofolate, prepared with [^{14}C]formaldehyde (or with the corresponding [6,7-^{3}H]tetrahydrofolate compounds), isolated by precipitation with ethanol and salt, digested to 3'-nucleotides, and chromatographed (1). Unlabeled methylenetetrahydrofolate was used to methylate tRNA in reaction mixtures containing 150 mCi ^3H_2O. The tRNA was precipitated several times from solution containing no label, digested to nucleotides, and chromatographed. From a similar reaction containing about 87% deuterium oxide, 12% glycerol, and 1% mercaptoethanol, by volume, tRNA was recovered and hydrolyzed in HCl (6 N, 180°C, 2hr); the bases were extracted with ethyl acetate/methanol/NH_4OH (18/1/1) and dried under N_2. Trimethylsilyl derivatives of the bases were prepared with BSTFA (with 20% pyridine; 150°C, 1 hr) and then separated and analyzed by gas chromatography-mass spectrometry on a 0.1% SE52 wall-coated-open-tubular column (0.325 mm x 10 m) directly coupled to a computerized Hitachi M-52 mass spectrometer (9).

RESULTS AND DISCUSSION

The purification and characterization of the enzyme, which have been described briefly (10), will be reported in detail elsewhere.

As previous data had indicated (1), the ribothymidine enzyme from *S. faecalis* is specific for tRNA(UΨC), lacking ribothymidine, and utilizes

Figure 1. Autoradiograph of chromatograms of 3'-nucleotides of tRNA methylated with a) methylene-[6-^{3}H]tetrahydrofolate or b) [^{14}C]methylene-[6-^{3}H]tetrahydrofolate. The center positions of the major nucleotides, which were located by visualization under ultraviolet light, are indicated by the white, lettered circles and the entire position of ribothymidylate by the black, broken line. Solvent systems as in reference 1.

TABLE I

INABILITY TO INCORPORATE LABEL FROM [6,7-^{3}H]TETRAHYDROFOLATE INTO RIBOTHYMIDINE

Substrates	[^{3}H]tetrahydrofolate	[^{3}H]ribothymidine
	mCi/mmol	mCi/mmol[a]
1. [^{14}C]methylene-[6-^{3}H]tetrahydrofolate + tRNA(UΨC)	1,200	30
2. [^{14}C]methylene-[6,7-^{3}H]tetrahydrofolate + tRNA(UΨC)	5.6	0.18
3. [^{14}C]methylene-[6,7-^{3}H]tetrahydrofolate + tRNA(UΨC)	10.5	0.80
4. methylene-[6,7-^{3}H]tetrahydrofolate + [*pyrimidyl*-2-^{14}C]tRNA(UΨC)	10.5	0.12

[a]Based on amount of ribothymidine as determined by carbon-14 incorporation

5,10-methylenetetrahydrofolate as the one-carbon donor. Reduced flavin is required by the pure enzyme *in vitro*. $FADH_2$, routinely generated nonenzymatically *in situ* from FAD and high concentrations of NAD(P)H, is superior to $FMNH_2$ and reduced riboflavin. NAD(P)H and other low potential reducing agents cannot overcome the flavin requirement (10).

Data obtained with impure enzyme (1) had suggested that hydrogen from the 6 position of tetrahydrofolate is not transferred to the one-carbon unit during the reduction of the methylene carbon of the folate cofactor to the methyl carbon of the modified residue, as it is in deoxythymidylate biosynthesis (6). These results were confirmed with pure enzyme.

As illustrated in Figure 1, little tritium was associated with ribothymidine synthesized with methylene-[6-^{3}H]tetrahydrofolate; however, carbon-14 was incorporated into ribothymidine from [^{14}C]methylene-[6-^{3}H]tetrahydrofolate. Using carbon-14 as a measure of ribothymidine, the specific activity of tritium label in ribothymidine was less than 3% that in tetrahydrofolate, as shown in Table I (experiment 1). Most, if not all, of the tritium appeared to be derived from degraded tetrahydrofolate (Figure 1a). Data from similar experiments, also summarized in Table I, indicated that little or no tritium from

[6,7-^{3}H]tetrahydrofolate is incorporated into ribothymidine. In a control reaction in experiment 3, *L. casei* thymidylate synthetase quantitatively incorporated tritium into deoxythymidylate (data not shown).

Since these data strongly suggested that hydrogen from neither the 6 nor 7 position of tetrahydrofolate is transferred to the one-carbon unit during ribothymidine formation, we speculated that the third hydrogen was probably derived from solvent, either directly or indirectly *via* an exchangeable position on the enzyme, $FADH_2$, or possibly tetrahydrofolate.

To examine this possibility, ribothymidine was synthesized in the presence of tritiated water. Following chromatography, the specific activity of ribothymidine was determined to be approximately 1 mCi/mmol, which was less than 10% that of hydrogen in the reaction mixture (13 mCi/mmol). In contrast to the experiments with [^{3}H]tetrahydrofolate, the tritium background was low; furthermore, essentially no label was found with uridylate. These data suggested that tritium was incorporated specifically into ribothymidine.

Since the amount of ribothymidine synthesized was in 50-fold excess over enzyme present, the low efficiency of incorporation could not be attributed to insufficient enzyme turnover. However, we calculated that hydrogen could come from solvent, if a tritium isotope effect were involved in the reaction (11).

To minimize ambiguities that might arise in such a case, the incorporation of hydrogen from solvent was examined by carrying out the reaction in solvent containing a high concentration of deuterium oxide (11) and analyzing the ribothymidine product by mass spectrometry.

Base analysis demonstrated that one ribothymidine residue was synthesized per tRNA molecule. The di-trimethylsilyl derivative of thymine from a control sample of tRNA methylated in ordinary water displayed a mass spectrum identical to that published by McCloskey (12). However, as illustrated in Figure 2, the mass spectrum of the thymine derivative from the deuterium-containing reaction showed that the molecular ion "M" and fragment ions "M - CH_3" and "a" were shifted upwards one mass unit to *m/e* = 271, 256 and 114, respectively, demon-

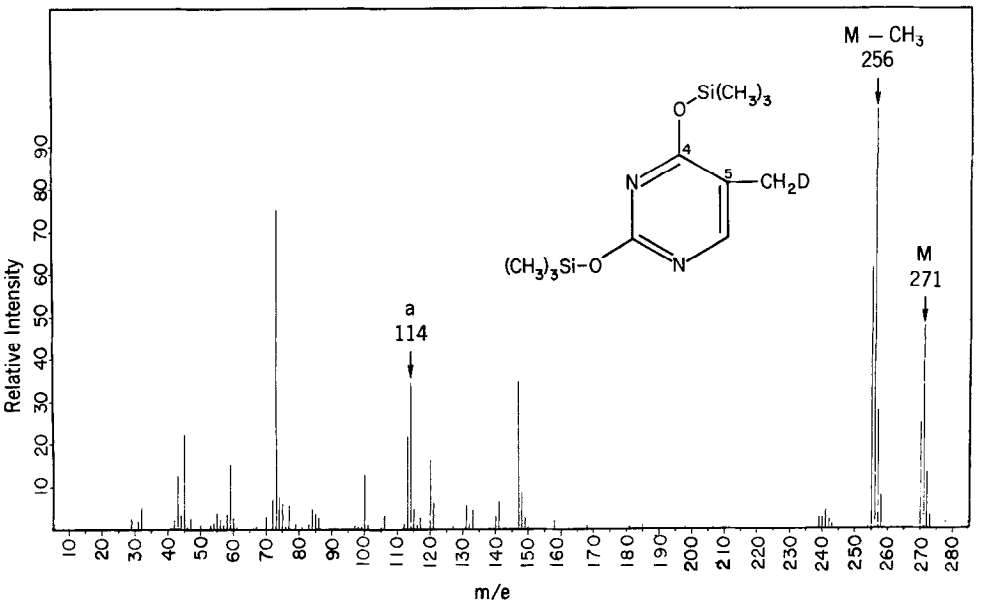

Figure 2. Mass spectrum of di-trimethylsilyl derivative of thymine synthesized in tRNA in the presence of deuterium oxide.

strating the incorporation of deuterium into these ions. The location of deuterium in the methyl moiety at carbon-5 is established by the shift in mass of "a," a diagnostic pyrimidine fragment which has been shown to result from cleavage of "M - CH_3" across the 3-4 and 5-6 bonds and to contain carbons 4 and 5 and their substituents (an O-dimethylsilyl and the methyl moiety, respectively) (12). No deuterium (<1%) was incorporated into uracil (not shown).

Deuterium was incorporated into about 65% of the ribothymidine residues; the failure of all ribothymidine residues to acquire deuterium is consistent with the presence of about 6% hydrogen in the reaction mixture (from residual water in the enzyme preparation and exchangeable positions on glycerol and mercaptoethanol) along with a deuterium isotope effect (11).

In view of our findings, we propose that the formation of ribothymidine in the tRNA of *S. faecalis*, and probably *B. subtilis* and *B. cereus*, occurs *via* the reaction shown in Figure 3, in which 5,10-methylenetetrahydrofolate serves as the one-carbon donor, but not as the source of the two electrons and hydrogen necessary for the reduction of the methylene carbon. The third hydrogen of

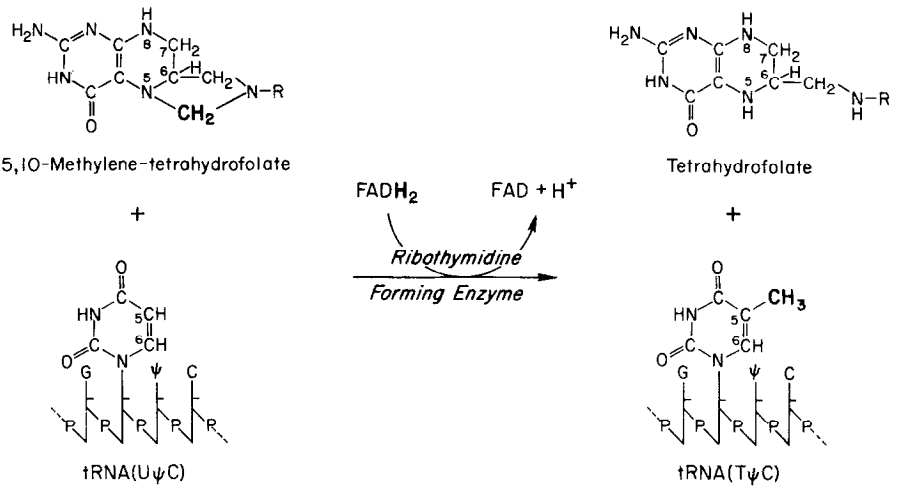

Figure 3. Proposed reaction for biosynthesis of ribothymidine in the tRNA of *S. faecalis*.

the methyl moiety of ribothymidine, *unlike* that of deoxythymidylate, is derived from solvent either directly or indirectly, as implied in the figure.

The identity of the reducing agent involved directly in the reaction remains to be established. It has not been possible to date to determine if tetrahydrofolate or dihydrofolate is the product of the reaction due, in part, to the low activity of the pure enzyme (0.1 μmol/min/mg) and the requirement for a large excess of methylenetetrahydrofolate in the *in vitro* reaction. The specific requirement for reduced flavin, rather than any low potential reducing agent, implies that flavin is uniquely involved in the reaction. The enzyme requires FAD for stability, suggesting that the enzyme may be a flavoprotein. We have considered that exogenous flavin may be serving in a non-physiological manner to regenerate reduced enzyme (10). These aspects are currently under investigation.

In summary, although 5,10-methylenetetrahydrofolate serves as the one-carbon donor in ribothymidine formation in *S. faecalis* tRNA, reduced flavin, not tetrahydrofolate, appears to be the effective reducing agent. The third hydrogen atom of the methyl moiety is derived from solvent, either directly or

indirectly *via* added $FADH_2$ or enzyme-bound flavin or perhaps *via* another group or amino acid residue on the enzyme.

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