JOURNAL OF BACTERIOLOGY, Jan. 1990, p. 252–259
0021-9193/90/010252-08$02.00/0

Vol. 172, No. 1

Chorismic Acid, a Key Metabolite in Modification of tRNA

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Received 14 April 1989/Accepted 2 October 1989

Chorismic acid is the common precursor for the biosynthesis of the three aromatic amino acids as well as for four vitamins. Mutants of *Escherichia coli* defective in any of the genes involved in the synthesis of chorismic acid are also unable to synthesize uridine 5-oxyacetic acid (cmo^5U) and its methyl ester ($mcmo^5U$). Both modified nucleosides are normally present in the wobble position of some tRNA species. Mutants defective in any of the specific pathways leading to phenylalanine, tyrosine, tryptophan, folate, enterochelin, ubiquinone, and menaquinone have normal levels of cmo^5U and $mcmo^5U$ in their tRNA. The presence of shikimic acid in the growth medium restores the ability of an *aroD* mutant to synthesize cmo^5U, while *O*-succinylbenzoate, which is an early intermediate in the synthesis of menaquinone, does not. Thus, chorismic acid is a key metabolite in the synthesis of these two modified nucleosides in tRNA. The absence of chorismic acid blocks the formation of cmo^5U and $mcmo^5U$ at the first step, which might be the formation of 5-hydroxyuridine. This results in an unmodified U in the wobble position of $tRNA_1^{Val}$ and in most of the tRNAs normally containing cmo^5U and $mcmo^5U$. Since cmo^5U and $mcmo^5U$ are synthesized under anaerobic conditions, the formation of these nucleosides does not require molecular oxygen. One of the carbon atoms of the side chain, —O—CH_2—COOH, originates from the methyl group of methionine. The other carbon atom does not originate directly from the C-1 pool, from the carboxyl group of methionine, or from bicarbonate. This metabolic link between intermediary metabolism and translation also exists for another member of the family *Enterobacteriaceae*, *Salmonella typhimurium*, as well as for the distantly related gram-positive organism *Bacillus subtilis*.

tRNA from all organisms contains a set of modified nucleosides, which are derivatives of the normal nucleosides. The formation of these occurs, with only two exceptions, on the polynucleotide level, i.e., after the formation of the primary transcript (6, 7). Thus, the modifying enzymes are involved in the maturation process of tRNA and are an integral part of the biosynthesis of tRNA. Two such modified nucleosides are uridine 5-oxyacetic acid (cmo^5U) and its methyl ester ($mcmo^5U$) (see Fig. 1), which are both present in the wobble position (position 34) of $tRNA_1^{Val}$, $tRNA^{Ser}$, $tRNA_1^{Pro}$, $tRNA^{Thr}$, and $tRNA^{Ala}$ from gram-negative bacteria. Corresponding tRNAs from gram-positive organisms have 5-methoxyuridine (mo^5U), which also might be an intermediate in the synthesis of cmo^5U in gram-negative organisms (25, 31; this study).

We previously established a link between intermediary metabolism and tRNA modification (5). All mutants defective in the common pathway for aromatic amino acids (Fig. 1; *aroB*, *aroD*, *aroE*, *aroA*, and *aroC*) are deficient in cmo^5U and $mcmo^5U$ in their tRNA. The presence of shikimic acid in the growth medium restores the synthesis of cmo^5U and $mcmo^5U$ in an *aroD* mutant but not in an *aroC* mutant. No metabolites in the specific pathways leading to tyrosine, phenylalanine, tryptophan, ubiquinone, enterochelin, and folate, known to branch out from chorismic acid, are involved in the modification. It was concluded that chorismic acid itself or a metabolite in the biosynthesis of menaquinone must play a key role in the formation of cmo^5U and $mcmo^5U$ in the tRNA (5). These two modified nucleosides are not found in the tRNAs specific for the aromatic amino acids (see above). Therefore, there is no direct relationship between their synthesis and the tRNAs specific for tyrosine, tryptophan, and phenylalanine. The function of these modified nucleosides in the tRNA may still in some way be coupled to the metabolism of aromatic amino acids, and it is thus necessary to know the molecular mechanism behind this metabolic link between the synthesis of aromatic amino acids and tRNA modification. In this study we ruled out an involvement of any known metabolite in the biosynthesis of menaquinone and suggest that chorismic acid itself or a metabolite in a hitherto unknown pathway from chorismic acid is involved in this modification of tRNA. This metabolite or chorismic acid is most likely required at the first step in the formation of cmo^5U. Furthermore, this link between intermediary metabolism and translation is also present in the closely related bacterium *Salmonella typhimurium* and also in the distantly related gram-positive organism *Bacillus subtilis*.

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used: U, uridine; m^5U, methyluridine (ribothymidine); C, cytidine; G, guanosine; A, adenosine; cmo^5U, uridine 5-oxyacetic acid; $mcmo^5U$, methyl ester of cmo^5U; mo^5U, 5-methoxyuridine; ho^5U, 5-hydroxyuridine; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry.

Materials. L-[*methyl*-^{14}C]methionine, L-[*methyl*-^{3}H]methionine, L-[1-^{14}C]methionine, [^{14}C]valine, [5-^{3}H]uracil, [2-^{14}C]uracil, and [^{14}C]uridine were from Amersham International PLC, Amersham, United Kingdom. [G-^{14}C]shikimic acid was from Dupont, NEN Research Products, Boston,

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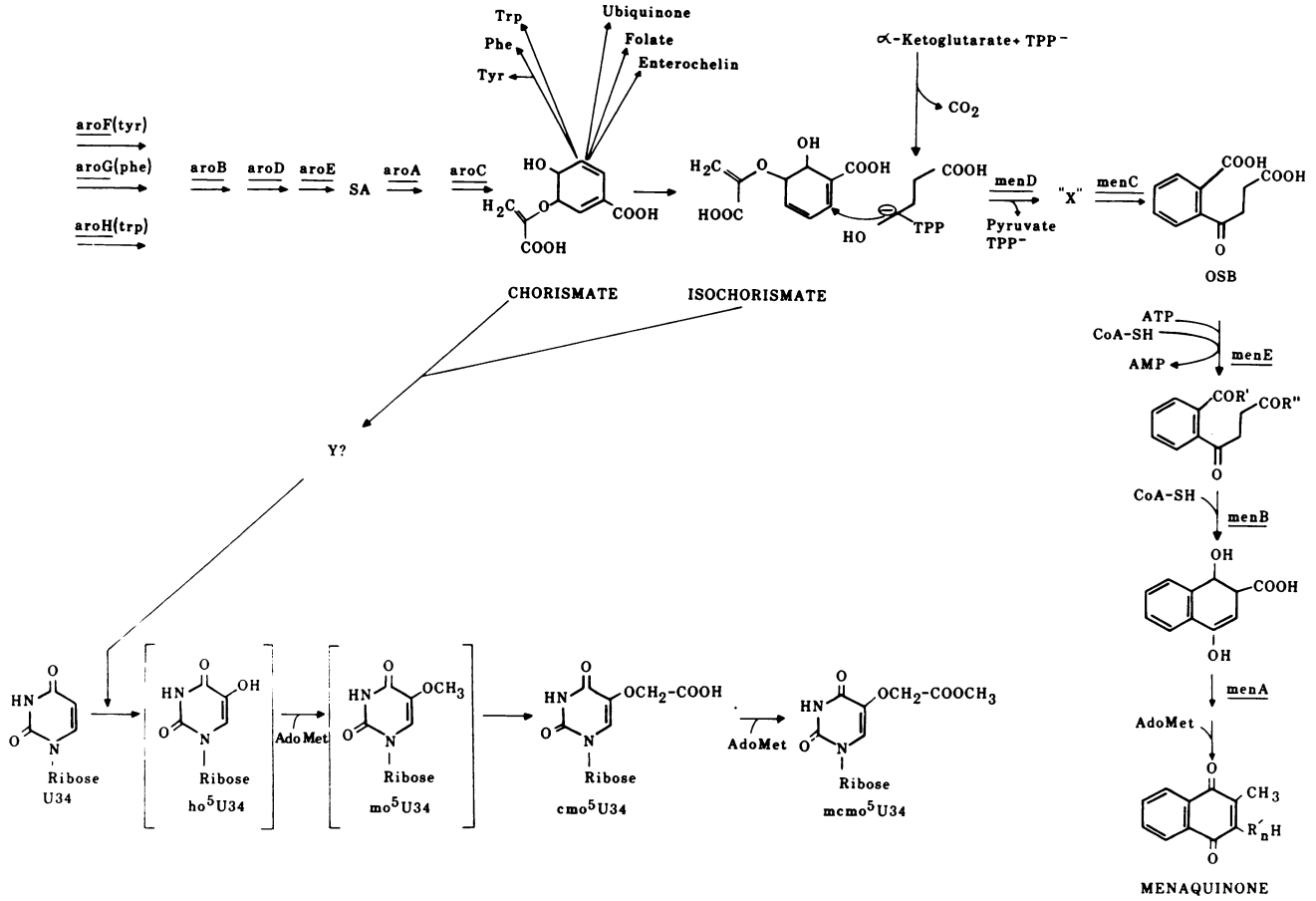

FIG. 1. Metabolic link among the syntheses of chorismic acid, menaquinone, and the two modified nucleosides (cmo^5U and $mcmo^5U$) present in the wobble position in some tRNAs. Y? denotes a possible derivative of chorismic acid. Intermediates ho^5U and mo^5U (in brackets) are postulated (see Discussion) and have not been identified. The menaquinone biosynthetic pathway is modified from Bentley and Meganathan (3). SA, Shikimic acid; TPP^-, thiamine PP_i; "X," unidentified intermediate in the conversion of isochorismate to *O*-succinylbenzoate (OSB); CoA-SH, coenzyme A; COR', COOH; COR", Co-CoA; R'_nH, side chain; AdoMet, *S*-adenosylmethionine.

Mass. Tetracycline, phenoxyacetic acid-*N*-hydroxysuccinimide ester, and potassium morpholinopropanesulfonate were from Sigma Chemical Co., St. Louis, Mo. DEAE-cellulose DE22 was from Whatmann Ltd., Springfield Mill, Maidstone, Kent, United Kingdom. Sephadex G-25 was from Pharmacia, Uppsala, Sweden.

Bacteria and phages. The *E. coli* K-12, *S. typhimurium*, and *B. subtilis* strains used in this study are listed in Table 1. For bacteriophage P1 transduction, P1 *vir*607H, kindly provided by L. Isaksson, Department of Microbiology, Uppsala University, Uppsala, Sweden, was used.

Growth media and cultivation. For genetic experiments, medium E, described by Vogel and Bonner (33), was supplemented with 0.2% glucose, 1 μg of thiamine per ml, 25 μg of the bases per ml, and 25 μg of the L isomer of the required amino acid per ml. The complete medium used was LB, described by Bertani (4) and supplemented with medium E and 0.2% glucose. For preparation of radioactive labeled RNA, cells were grown in rich MOPS medium, which contains 20 amino acids, two purines, two pyrimidines, and five vitamins (26). When labeling was done with [^{14}C]uracil or [^{14}C]uridine, cytosine was omitted. Anaerobic growth in rich MOPS medium took place in a glove box, which contained 85% N_2, 10% H_2, and 5% CO_2 and was continuously circulated through a catalyst of palladium-coated aluminum pellets (type D; Engelhard & Co., Västra Frölunda, Sweden) (35). The humidity was kept at 20 to 30% saturation. A 0.1% (wt/vol) solution of benzyl viologen in 50 mg of potassium phosphate buffer (pH 7.0) was reduced when stored in the box. Media were prepared outside the box but stored in the box for at least 18 h before use. The absence of O_2 in the box was routinely tested with the strictly aerobic bacterium *Pseudomonas aeruginosa*.

Uptake of [^{14}C]shikimic acid. The uptake of [^{14}C]shikimic acid was measured as described by Brown and Doy (10). Cells were grown at 37°C in basal medium 56 (10) containing complete aromatic supplementation. At a cell density of about 2×10^8 cells per ml, cells were filtered, washed with 10 volumes of medium lacking the aromatic supplements, and suspended in the same medium. Following incubation of the culture for 50 min, when there was no mass increase, samples were added to a flask containing shikimic acid (2 mCi/mmol; 10 mM; time zero). Samples (1 ml) were removed at various times, filtered, washed with 20 ml of basal medium, and counted in a scintillation counter.

Preparation of tRNA for two-dimensional thin-layer chromatography. Cells were grown in rich MOPS medium containing L-[*methyl*-^{14}C]methionine (0.07 mM; 55 μCi/μmol), [^{14}C]uracil (0.022 mM; 55 μCi/μmol), or [^{14}C]uridine (0.015 mM; 488 μCi/μmol). Cells were harvested as overnight

TABLE 1. *E. coli* K-12, *S. typhimurium*, and *B. subtilis* strains

Strain	Relevant genotype or phenotype	Source or reference
E. coli		
BW113	HFr P4X *metB* λ^s λ^-	
GRB107	Hfr P4X *metB aroD45*::Tn*10* λ^s λ^-	This study
GRB108	Hfr P4X *metB* Aro^+; spontaneous revertant of GRB107	This study
GB707	Hfr P4X *argHI trmA5 thiA aroD*::Tn*5*	5
GB711	Hfr P4X; $argH^+$; transductant of GB707 which also had become $trmA^+$ $thiaA^+$	This study
JRG860	F^- *gal trpA trpR iclR rpsL menC3*	J. Guest
JRG916	F^- *gal trpA trpR iclR rpsL nalA menD5*	J. Guest
JRG962	F^- *gal trpA trpR iclR rpsL nalA menB*	J. Guest
PL2024	F^- *gal trpA trpR iclR rpsL*	J. Guest
S. typhimurium		
LT2	Wild type	J. Roth
aroA64	*aroA64*	K. E. Sanderson
aroB74	*aroB74*	K. E. Sanderson
aroC5	*aroC5*	K. E. Sanderson
aroD85	*aroD85*	K. E. Sanderson
TT1452	*aroA551*::Tn*10*	J. Roth
aroE36	*aroE36*	B. Stocker
TT10799	*aroD558*::*Mu dA*	J. Roth
B. subtilis		
1A613	*trpC2 aroBC84*::Tn*917*	D. R. Ziegler
1L5	*trpC2*	D. R. Ziegler

cultures or as logarithmically growing cultures at a density of about 6×10^8 cells per ml. tRNA was prepared as described earlier (5). The method involves freezing and thawing of the cells, phenol extraction, and separation of rRNA and tRNA with 2 M LiCl. tRNA was digested to nucleosides and analyzed by two-dimensional thin-layer chromatography as described by Rogg et al. (29), except that the first solvent system was developed for 40 h instead of the recommended 18 h. This allowed the separation of ho^5U from dihydrouridine, pseudouridine, and other [^{14}C]uracil-labeled components. In some cases, the chromatography was performed by another two-dimensional system which used isopropanol-concentrated ammonium hydroxide-H_2O (7:1:2) as solvent 1 in the first dimension and isopropanol–1% ammonium sulfate (2:1) as solvent 2 in the second dimension. This two-dimensional thin-layer chromatography system also separated ho^5U from other [^{14}C]uracil-labeled compounds.

Occasionally, ho^5U was found, and its identification was based on the fact that the radioactive compound denoted ho^5U comigrated in these four solvents with the marker ho^5U. Since ho^5U migrated close to dihydrouridine and pseudouridine, which are both present in much larger amounts than ho^5U, the estimated level of ho^5U was always corrected for the radioactivity present in this area of the chromatogram from an analysis of tRNA from the control (Aro^+) strain. Furthermore, a distinct radioactive spot was observed comigrating with the UV marker, and this radioactive spot was never observed in tRNA from Aro^+ cells. No or very little tailing of radioactivity from dihydrouridine was observed; this might have incorrectly been counted as ho^5U.

Since the amount of ho^5U varied from experiment to experiment, we tested whether this variability was due to chemical instability. A sample of ho^5U was treated in the same way as tRNA; no indication of chemical instability of ho^5U during the treatment was observed.

Purification of $tRNA_1^{Val}$. Crude tRNA was prepared (2) from strain GB711 (*aroD*::Tn*5*), cultivated with or without shikimic acid. The presence of shikimic acid in the growth medium restores the ability of an *aroD* mutant to synthesize cmo^5U (5). $tRNA_1^{Val}$ was separated from $tRNA_2^{Val}$ on benzoylated DEAE-cellulose (18). Crude tRNA (200 to 400 mg) dissolved in 30 ml of 0.3 M NaCl–0.01 M $MgSO_4$ was applied to a 150-ml column of benzoylated DEAE-cellulose and eluted with a linear gradient of NaCl (1,000 to 2,750 ml of 0.3 M NaCl–0.01 M $MgSO_4$ in the mixing chamber and 1,000 to 2,750 ml of 1 M NaCl–0.01 M $MgSO_4$ in the reservoir). $tRNA_1^{Val}$ was further purified by chromatography on benzoylated DEAE-cellulose after phenoxyacetylation (17). The pure $tRNA_1^{Val}$ was deacylated in 0.1 M Na_2CO_3 and desalted on a Sephadex G-25 column operating with water. Fractions containing tRNA were pooled, lyophilized, and dissolved in distilled water. The concentration of $tRNA_1^{Val}$ was calculated from its optical density; 1 A_{260} unit equals 1 μmol of $tRNA_1^{Val}$.

Analysis of modified nucleosides of $tRNA_1^{Val}$ by HPLC. Analysis of modified nucleosides by HPLC was performed essentially as described by Gehrke et al. (15, 16) with μBondapak C_{18} (600 by 4 mm) columns in 0.01 M $NH_4H_2PO_4$ (pH 5.1, pH 4.8, or pH 6.3, as needed). Different concentrations of methanol were used, and the column was operated at 35.5°C with a flow rate of 1 ml/min. In a later stage of the investigation, a Radial PAK C_{18} column in a Radial Compression System 100 was used. In the analysis of modified nucleosides of $tRNA_1^{Val}$, the complex gradient of Buck et al. (11) was also used.

Determination of the sequence of purified $tRNA_1^{Val}$ from Aro^+ and Aro^- strains. Purified $tRNA_1^{Val}$ from strain GB711 (*aroD*::Tn*5*), cultivated with or without the addition of shikimic acid, was sequenced as described by Keith et al. (22), except that the two-dimensional thin-layer chromatographic system of Nishimura (27) was used to identify the modified nucleotides. In this system, ho^5U-5′-phosphate, cmo^5U-5′-phosphate, and U-5′-phosphate are well separated. Synthetic markers of ho^5U-5′-phosphate and cmo^5U-5′-phosphate (kind gifts from K. Murao, Iichi Medical School, Tokyo, Japan, and S. Nishimura, National Cancer Center Research Institute, Tokyo, Japan, respectively) al-

TABLE 2. In vivo level of cmo^5U in the tRNA of *E. coli* strains carrying mutations in different genes involved in the synthesis of menaquinone

Mutation	Addition to rich MOPS medium	cmo^5U/tRNA (mol/mol)
None (wild type)		0.12
aroD::Tn*5*		<0.01
aroD::Tn*5*	Shikimic acid	0.12
aroC355	Shikimic acid	<0.01
aroD::Tn*5*	Vitamin K_3	<0.01
aroD::Tn*5*	*O*-Succinylbenzoate	0.03
menC		0.15
menD		0.20
menB		0.21

lowed us to directly analyze the presence of both of these nucleotides in the anticodon position of $tRNA_1^{Val}$.

Hydrolysis of tRNA for LC-MS analysis. Quantities of 100 μg of tRNA were hydrolyzed in 50 μl of 0.01 M ammonium acetate (pH 5.3) by nuclease P1 (2 U/100 μg of tRNA) for 8 h at 37°C. Two microliters of 1 M NH_4OH and alkaline phosphatase (0.5 U/1,200 μg of tRNA) were added, and incubation was continued at 37°C for 12 h. Aliquots were directly examined by HPLC or combined LC-MS.

LC-MS. Chromatography was carried out with a model 322M liquid chromatograph (Beckman Instruments, Inc., Fullerton, Calif.) with a 3μ Ultrasphere ODS column (4.6 by 75 mm) and a mobile phase of 2 ml of 0.25 M ammonium acetate (pH 6) per min. The mass spectrometer consisted of a noncommercial quadrupole mass analyzer, as previously detailed (14), which operates on the thermospray ionization principle (32) and utilizes a Vestec Corp. (Houston, Tex.) probe and temperature controller. Vaporizer exit temperatures were maintained at 240°C, and the ion source temperature was controlled so as to maximize thermospray ionization yield, which afforded a vapor temperature at the point of ion sampling of 292°C. All measurements were made by selected ion monitoring with the mass spectrometer under the control of a model 1050 data system (Teknivent, St. Louis, Mo.). Samples corresponding to approximately 2 μg of tRNA (1.5 μg of nucleosides) were injected for each LC-MS analysis. A description of the instrument and technique for analysis of nucleosides in hydrolysates of tRNA was reported previously (14).

RESULTS

Chorismic acid is a key metabolite in the synthesis of cmo^5U. We previously showed that chorismic acid or a metabolite in the synthesis of menaquinone is required for the biosynthesis of cmo^5U and $mcmo^5U$ in tRNA (5). To investigate whether some intermediate in the synthesis of menaquinone is involved in the modification of tRNA, we determined the level of cmo^5U in the tRNA of mutants defective in the biosynthesis of menaquinone (Table 2). Mutants defective in *menC*, *menD*, and *menB* genes had normal levels of cmo^5U. Furthermore, *O*-succinylbenzoic acid is known to be taken up by *E. coli* and to phenotypically suppress *menC* and *menD* mutants (13, 19, 20, 37). Supplying this compound in the growth medium of an *aroD* mutant did not completely restore the synthesis of cmo^5U in tRNA (Table 2). These results show that no metabolite following *O*-succinylbenzoic acid is involved in the synthesis of cmo^5U in tRNA. The small amount of cmo^5U made upon the addition of *O*-succinylbenzoic acid (0.03 mol/mol of tRNA) may indicate some reversibility between this metabolite and chorismic acid. Therefore, chorismic acid itself or a metabolite in a hitherto unknown biosynthetic pathway is required for the formation of cmo^5U in tRNA.

The metabolic block in Aro^- mutants is at the first step in the synthesis of cmo^5U. Two possible routes for cmo^5U synthesis have been suggested (25). Either the synthesis starts with hydroxylation producing ho^5U followed by methylation and carboxylation or alternatively by direct acetylation of ho^5U. Thus, the metabolic pathway may be

$$U \xrightarrow{OH} ho^5U \xrightarrow{CH_3} mo^5U \xrightarrow{CO_2} cmo^5U \text{ or}$$

$$U \xrightarrow{OH} ho^5U \xrightarrow{CH_2COOH} cmo^5U$$

The fact that the methyl group from methionine is a precursor to cmo^5U suggests the first alternative (5). To investigate how a mutation in the *aro* pathway blocks the biosynthesis of cmo^5U and $mcmo^5U$, we analyzed what constituent is present in position 34 in those tRNAs normally possessing these modified nucleosides. We did this analysis at two levels: analysis of bulk tRNA and analysis of purified $tRNA_1^{Val}$, which is known to normally contain cmo^5U (24).

Strain GRB107 (Aro^-) and strain GRB108 (Aro^+) were grown in rich MOPS medium containing [^{14}C]uracil or [^{14}C]uridine. Bulk tRNA was purified and degraded to nucleosides, and the mixture was analyzed by thin-layer chromatography. This procedure was done earlier, and no extra compound was observed in the tRNA from an Aro^- mutant (5). However, by developing the first system for a longer time, we were able to separate ho^5U from pseudouridine. We also used another two-dimensional chromatography system to investigate the possible presence of ho^5U in tRNA from the Aro^- strain. When m^5U was used as an internal standard, the level of cmo^5U and $mcmo^5U$ in the Aro^+ strain was 0.15 mol/mol of m^5U. The level of ho^5U varied from 0 to 0.02 mol/mol of m^5U, which is 0 to 15% of the expected level assuming the biosynthetic pathway to be as simple as stated above (data not shown). The migration behavior of the suggested intermediate mo^5U was such that its position in the chromatogram was well separated from those of other nucleosides, and no such compound was observed in tRNA from the Aro^- mutant. HPLC analysis of bulk tRNA as well as $tRNA_1^{Val}$ (see below) with different methanol concentrations (0, 1, 2.5, and 6%), different pHs (5.1 and 6.3), and the conditions devised by Buck et al. (11) did not reveal any Aro^--specific nucleoside derivative (results not shown). Thus, no Aro^--specific compound other than the occasional presence of small amounts of ho^5U was observed when bulk tRNA was analyzed.

$tRNA_1^{Val}$ contains cmo^5U in position 34 (24). In a specific search for the two potential intermediates, ho^5U and mo^5U, that might be present in $tRNA_1^{Val}$ in Aro^- cells, nucleosides from purified $tRNA_1^{Val}$ were analyzed by LC-MS. The resulting chromatograms, obtained by both UV and mass spectometric detection, are shown in Fig. 2. The ions monitored corresponded to protonated molecular species (MH^+) for ho^5U, mo^5U and, as an internal control, m^5U (*m/z* 261, *m/z* 275, and *m/z* 259, respectively). The chromatographic system used was chosen to minimize interference in the ho^5U channel (*m/z* 261) from minor ions from cytidine, with which ho^5U coelutes. Under these conditions, no signals were obtained in the ho^5U channel when cytidine alone was analyzed (data not shown). The selected ion recordings in Fig. 2A showed responses and expected elu-

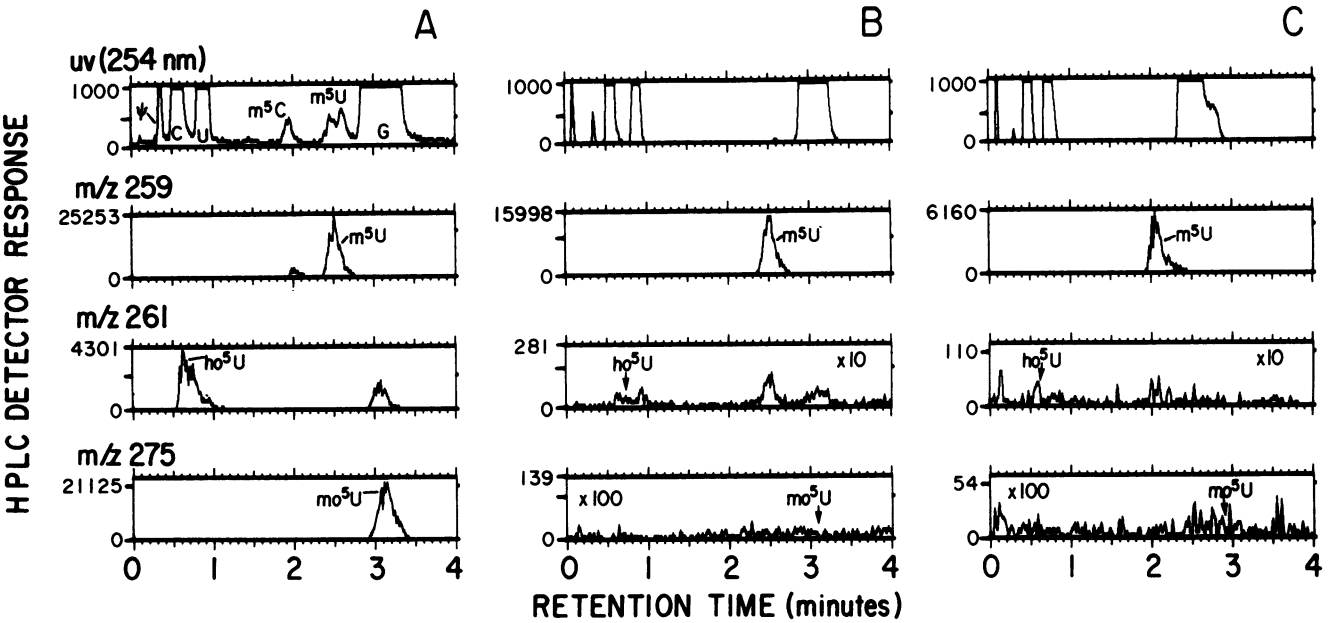

FIG. 2. UV absorbance and selected ion chromatograms from the LC-MS analysis of enzymatic hydrolysis products of tRNA. (A) $tRNA_f^{Met}$ spiked with one equivalent each of ho^5U and mo^5U as a model to calibrate ion current response and elution times for m^5U, ho^5U, and mo^5U. m^5C, 5-Methylcytidine. (B) $tRNA_1^{Val}$ from strain GB711 grown in the presence of shikimic acid. (C) $tRNA_1^{Val}$ from strain GB711 grown in the absence of shikimic acid. For each panel, the top section shows UV absorbance detection at A_{254}. The lower sections represent MH^+ ions for m^5U (*m/z* 259), ho^5U (*m/z* 261), and mo^5U (*m/z* 275). The arrows in panels B and C mark the expected elution positions of ho^5U and mo^5U in each experiment. Absolute ion intensity values for each mass channel are listed on the ordinates. On the basis of the peak area for m^5U (1 mol per tRNA) in each analysis, ordinates in panels B and C for *m/z* 261 and *m/z* 275 were adjusted to provide (with amplification factors of 10 and 100) sensitivity scales equivalent to 1 mol of ho^5U and mo^5U, respectively.

tion positions of m^5U, ho^5U, and mo^5U relative to the major ribonucleosides (UV channel, top section). $tRNA_1^{Val}$ from Aro^- cells grown in the absence or presence of shikimic acid did not contain the two potential intermediates ho^5U and mo^5U (less than 0.01% of the level of m^5U). The LC-MS experiments strongly suggested that $tRNA_1^{Val}$ from cells grown in the absence of shikimic acid contains an unmodified U in the wobble position. Furthermore, direct sequencing of purified $tRNA_1^{Val}$ by the postlabeling technique revealed only unmodified U-5′-phosphate in position 34 in tRNA from cells grown in the absence of shikimic acid, while cmo^5U was present in control tRNA (data not shown). Taken together, all our results are consistent with the conclusion that the block in the Aro^- mutant is at the first step in the biosynthesis of cmo^5U and $mcmo^5U$, resulting in an unmodified U. The occasional presence of ho^5U in bulk tRNA but not in $tRNA_1^{Val}$ may have been due to its presence in small amounts in some of the other tRNA chains that normally contain cmo^5U and $mcmo^5U$.

Metabolic routes in the biosynthesis of cmo^5U and $mcmo^5U$. Since ho^5U was occasionally observed in tRNA in Aro^- cells, the first step in the synthesis of cmo^5U and $mcmo^5U$ might be a hydroxylation reaction. Many hydroxylation reactions require molecular oxygen as a cofactor. cmo^5U is

TABLE 3. Relative amounts of cmo^5U in the tRNAs of different *E. coli* and *S. typhimurium* strains grown in the presence of different radioactive metabolites under various conditions

Strain	Growth medium	Growth condition	Radioactive component	cpm of cmo^5U/ cpm of m^5U
E. coli				
BW113	Rich MOPS	$+O_2$	[*methyl*-^{14}C]methionine	0.12
BW113	Rich MOPS	$-O_2$	[*methyl*-^{14}C]methionine	0.12
BW113	Rich MOPS	$+O_2$	[*methyl*-^{3}H]methionine	0.01
GRB108	Rich MOPS	$+O_2$	[2-^{14}C]uracil	0.11
BW113	Rich MOPS	$+O_2$	$H^{14}CO_3$	0.17
S. typhimurium				
LT2	Rich MOPS	$+O_2$	[*methyl*-^{14}C]methionine	0.12
aroA64	Rich MOPS	$+O_2$	[*methyl*-^{14}C]methionine	<0.01
aroA64	Rich MOPS + shikimic acid	$+O_2$	[*methyl*-^{14}C]methionine	0.03
aroB74	Rich MOPS	$+O_2$	[*methyl*-^{14}C]methionine	<0.01
aroB74	Rich MOPS + shikimic acid	$+O_2$	[*methyl*-^{14}C]methionine	0.02
TT1452	Rich MOPS	$+O_2$	[*methyl*-^{14}C]methionine	<0.01
aroE36	Rich MOPS	$+O_2$	[*methyl*-^{14}C]methionine	0.04

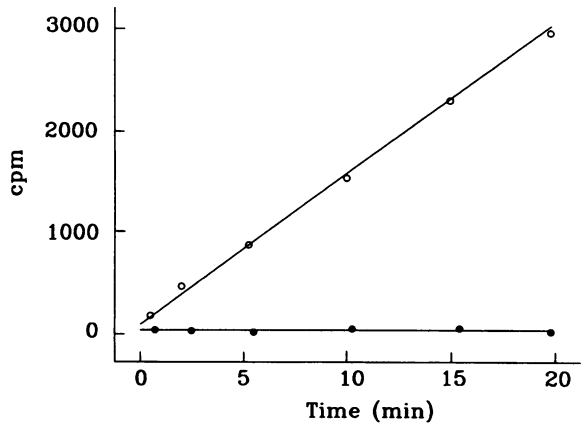

FIG. 3. Uptake of [^{14}C]shikimic acid by *E. coli* GRB107 (*aroD45*::Tn*10*; ○) and *S. typhimurium* TT10779 (*aroD558*::Mu dA; ●).

made under anaerobic conditions (Table 3); therefore, another hydroxylation mechanism must exist if the suggested pathway operates.

The methyl group from methionine is the origin of at least one of the two carbon atoms in the side chain of cmo^{5}U, since this modified nucleoside is radioactively labeled when cells are grown in rich MOPS medium containing L-[*methyl*-^{14}C]methionine (5). This medium contains all amino acids, including glycine and serine, which are known precursors to the C-1 pool as well as purine bases and *p*-aminobenzoic acid. The same cmo^{5}U/m^{5}U ratio was obtained after growth in the presence of [2-^{14}C]uracil or L-[*methyl*-^{14}C]methionine, demonstrating that only one of the two carbon atoms in the —O—CH_2—COOH side chain of cmo^{5}U originates from methionine (Table 3). No specific label was found in cmo^{5}U or mcmo^{5}U in tRNA from cells grown in medium containing L-[1-^{14}C]methionine (data not shown). Furthermore, the other C atom did not originate from bicarbonate (known C-atom donor in the biosynthesis of another modified nucleoside, N^6-threonyladenosine) (Table 3) or from the C-1 pool (data not shown). When cells were grown in the presence of L-[*methyl*-^{3}H]methionine, only 10% of the expected radioactivity was stably incorporated into cmo^{5}U (Table 3). (8). Thus, the hydrogen atoms of the methyl group of L-methionine participate in some reaction during the formation of the side chain of cmo^{5}U and are subsequently lost from cmo^{5}U.

A metabolic link between chorismic acid and tRNA modification also exists in *S. typhimurium* and possibly in *B. subtilis*. The levels of cmo^{5}U and mcmo^{5}U in tRNA of different mutants in the common aromatic pathway of *S. typhimurium* LT2 were analyzed. All *aro* mutants tested were deficient in cmo^{5}U (Table 3). However, unlike in *E. coli*, the addition of shikimic acid to the growth medium did not allow strain aroB74 to synthesize cmo^{5}U (Table 3), because *S. typhimurium* cannot take up this compound (Fig. 3). The tRNAs specific for valine, alanine, threonine, and serine from gram-negative organisms have cmo^{5}U and mcmo^{5}U in the wobble position. Corresponding tRNAs from gram-positive organisms have mo^{5}U, which might be an intermediate in the synthesis of cmo^{5}U and mcmo^{5}U in gram-negative organisms (25) (Fig. 1). To establish whether the biosynthetic link between chorismic acid and tRNA modification has been conserved, we analyzed the level of mo^{5}U in tRNA from *B. subtilis* 1L5 (Aro$^+$) and 1A613 (Aro$^-$) by two methods. For the first method, LC-MS analysis was performed as for Fig. 2 and as described in Materials and Methods. Levels of mo^{5}U (relative to the level of mo^{5}U in the wild-type strain [Aro$^+$] with m^{5}U as an internal standard) were 31 and 38% in experiments 1 and 2 (two independent tRNA preparations), respectively. For the second method, cells were grown in rich MOPS medium containing L-[*methyl*-^{14}C]methionine (58 mCi/mmol; 1.72 μM), tRNA was prepared from two independent cultures (experiments 1 and 2) as described by Vold (34) and in Materials and Methods and was digested to nucleosides as described by Rogg et al. (29), and the composition of methylated nucleosides was analyzed by HPLC (C. W. Gehrke and K. C. Kuo, submitted for publication). The radioactivity in the eluate was determined with a Radiomatic liquid scintillation counter. Levels of mo^{5}U (relative to the level of mo^{5}U in tRNA from the Aro$^+$ strain with m^{2}A and m^{6}A [they comigrate] as internal standards) were 27 and 47% in experiments 1 and 2, respectively. The radioactive peak denoted mo^{5}U had the same retention time as the synthetic marker mo^{5}U. These results indicated that a defect in the synthesis of chorismic acid in *B. subtilis* also decreased the level of mo^{5}U in tRNA by 65%. No other changes in tRNA modification were detected by comparing the UV profiles of the HPLC chromatograms (data not shown). Thus, the metabolic link between chorismic acid and tRNA modification exists not only in members of the family *Enterobacteriaceae* but possibly also in the distantly related gram-positive organism *B. subtilis*.

DISCUSSION

We have shown that chorismic acid is a key metabolite in the modification of tRNA (Fig. 1). The absence of chorismic acid most probably blocks the formation of cmo^{5}U and mcmo^{5}U in the first step of their biosynthesis. This metabolic block therefore results in an unmodified U in the wobble position of $tRNA_1^{Val}$ and most likely also in most other tRNAs normally containing these modified nucleosides. ho^{5}U may be the first intermediate; if so, the hydroxylation reaction does not require molecular oxygen, since it also occurs under anaerobic conditions. Furthermore, only one carbon atom of the —O—CH_2—COOH side chain of cmo^{5}U originates from the methyl group of methionine. The second carbon atom does not originate either from bicarbonate or directly from the C-1 pool (Table 3), and its origin is so far unknown.

Figure 1 shows the biosynthetic pathway from chorismic acid to menaquinone. Chorismic acid is converted by the *menD* gene product to an unknown compound (X), which is then converted to *O*-succinylbenzoate (OSB) by the *menC* gene product. The level of cmo^{5}U in tRNA from a *menD* mutant is normal (Table 2). This would suggest that the metabolite required for the synthesis of cmo^{5}U, if part of the menaquinone pathway, must be before compound X. This conclusion is based on the assumption that the mutation in the *menD* gene is not leaky. The fact that *O*-succinylbenzoate did not fully restore the synthesis of cmo^{5}U in an *aroD*::Tn*5* mutant suggests that a metabolite after O-succinylbenzoate in the synthesis of menaquinone is not involved in the modification of tRNA. However, the partial restoration may indicate some reversibility between O-succinylbensoic acid and chorismic acid, although other possibilities cannot be ruled out at present. Since all mutations tested in the menaquinone pathway did not prevent the formation of cmo^{5}U, we favor the first possibility. Thus, if the metabolite is not part of the menaquinone pathway, it must be chorismic acid itself or a metabolite in a hitherto unknown metabolic

pathway from chorismic acid that is required for the formation of cmo^5U and $mcmo^5U$ in tRNA.

It is known that auxotrophic mutants of *E. coli* blocked before shikimic acid (*aroB*, *aroD*, or *aroE*) grow very poorly on shikimic acid as the sole aromatic supplement because of a poor uptake system for shikimic acid (10, 28). This poor transport of shikimic acid is sufficient to fully restore the synthesis of cmo^5U in tRNA in an *aroD* mutant (5). This observation suggests that only a low level of chorismic acid is required for the synthesis of cmo^5U, consistent with the low level of this modified nucleoside found in the cells. The block in the transport of shikimic acid is much more efficient in *S. typhimurium* than in *E. coli* (Fig. 3), which explains why the addition of shikimic acid to an *aroB* mutant of *S. typhimurium* does not suppress the synthesis of cmo^5U in tRNA (Table 3).

ho^5U was occasionally present in small amounts in bulk tRNA but not in $tRNA_1^{Val}$ from Aro^- mutants, possibly because of its presence in small amounts in some tRNA chains that normally contain cmo^5U and $mcmo^5U$. If so, ho^5U is an intermediate in the synthesis of cmo^5U. Alternatively, its occasional presence could be due to a side reaction occurring only in Aro^- cells. In this case, ho^5U would not be a true intermediate. Starvation of a *relA met* mutant of *E. coli* for methionine results in the accumulation of generally methyl-deficient tRNA (9). Purified $tRNA_1^{Val}$ from such cells contains ho^5U (25). This fact, together with our observation of the occasional occurrence of ho^5U in tRNA from Aro^- cells, is consistent with ho^5U being an intermediate in the biosynthesis of cmo^5U, although other alternatives cannot be ruled out.

We hypothesize that in the first step in the synthesis of cmo^5U, chorismic acid (or a derivative of it) stimulates the reaction(s), which may go through ho^5U as an intermediate. Without chorismic acid, this reaction(s) proceeds very slowly with some tRNAs as substrates but not at all with other tRNAs. The step following the formation of ho^5U may also require chorismic acid, since it was occasionally found in tRNA from Aro^- cells. In *B. subtilis*, mo^5U is present in the same position in the corresponding tRNA species as are cmo^5U and $mcmo^5U$ in *E. coli*. We showed that the synthesis of mo^5U in tRNA from *B. subtilis* is also sensitive to the perturbation in the metabolism of chorismic acid (see above). These facts suggest that mo^5U may also be an intermediate in the synthesis of cmo^5U (25). Irrespective of the possibility that ho^5U and mo^5U are intermediates in the formation of cmo^5U, our results strongly suggest that the first step in its biosynthesis requires chorismic acid, resulting in an unmodified U instead of cmo^5U in the wobble position in most, if not all, tRNAs.

tRNAs specific for valine, serine, proline, threonine, and alanine from eucaryotes, from gram-positive organisms, and from gram-negative organisms contain in the wobble position inosine, mo^5U, and cmo^5U and $mcmo^5U$, respectively (31). The presence of these modified nucleosides in this position increases the wobble capacity in such a way that the tRNAs not only read codons ending with A or G, according to the wobble hypothesis, but also read codons ending with U (12, 36). However, in these organisms there are also other tRNAs able to read codons ending with C or U. Therefore, it is not obvious why these modified nucleosides are present in tRNA. However, the same kinds of tRNAs from mitochondria and from two *Mycoplasma* species contain an unmodified U in the wobble position (1, 21, 23, 30). Besides these *Mycoplasma* species, the Aro^- mutants of *E. coli* and *S. typhimurium* are the only cell types that so far have been shown to contain an unmodified U in the wobble position (31; this study). In mitochondria, as in the *Mycoplasma* species, only one tRNA species decodes all four codons in most codon families. Therefore, a tRNA with an unmodified U in the wobble position in *E. coli* as well might be able to read codons ending with U but perhaps with a lower efficiency than tRNAs with a modified U in position 34. This would explain why these modified nucleosides are not essential for cell growth but might be involved in reading codons ending with U in certain codon contexts not so well read by the alternative tRNAs normally reading codons ending in C or U. In fact, an Aro^- mutant of *E. coli* that lacks cmo^5U and $mcmo^5U$ grows as much as 20% slower than Aro^+ cells in rich medium containing all metabolites synthesized from chorismic acid (data not shown). Thus, the presence of these modified nucleosides is important under some physiological conditions. Their synthesis might therefore be an example of how the degree of tRNA modification might control part of the intermediary metabolism (reviewed in reference 7). Exact knowledge of the molecular mechanism of the interlink, as well as the function of the modified nucleosides in tRNA, will be necessary to fully understand the evolution of such links between intermediary metabolism and translation.

ACKNOWLEDGMENTS

This work was supported by the Swedish Natural Science Research Council (grant B-BU2930), the Swedish Cancer Society (project 680), the Swedish Technical Board of Development, and the National Institutes of Health (Public Health Service grant GM 29812). One of us (Yvonne H. Jönsson) was supported by a short-term fellowship from the European Molecular Biology Organization to learn tRNA sequencing in the laboratory of G. Keith, Strasbourg, France.

The skillful technical assistance of K. Jacobsson, G. Jäger, M. Kimdahl, and A. Olsén is gratefully acknowledged. We are also grateful to J. Guest, Sheffield, England, for generous gifts of *O*-succinylbenzoate and strains carrying different *men* mutations. We are also grateful to K. Stråby, Umeå, Sweden, and M. Mahan, D. Roof, and D. Andersson, Salt Lake City, Utah, for critical reading of the manuscript.

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