Participation of Acetylpseudouridine in the Synthesis of a Peptide Bond in Vitro*

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Uracil, uridine, and pseudouridine were acetylated by refluxing in acetic anhydride, and the products of acetylation were incubated with a synthetic peptide (1–21) that corresponds to the N-terminal 21 amino acid residues of human myelin basic protein. Peptide bond formation, at the N° terminus in peptide 1–21, was obtained with acetyluracil and acetylpseudouridine, but not with acety luridin. Transfer of an acetyl group from acetyluracil and acetylpseudouridine depended on acetylation in the N-heterocycle. X-ray crystallographic analysis definitively established N-1 as the site of acetylation in acetyluracil. Mass spectrometry of the acetylation products showed that one acetyl group was transferred to peptide 1–21, in water, by either acetyluracil or acetylpseudouridine at pH 6.6. Release of the acetyl group by acylaminopeptidase regenerated peptide 1–21 (mass spectrometry) and automated sequencing (for five cycles) of the regenerated (deacetylated) peptide demonstrated that the N terminus was intact. The findings are discussed in the context of a possible role for pseudouridine in ribosome-catalyzed peptide transfer, with particular reference being made to similarities between the possible mechanism of acyl transfer by acetyluracil/pseudouridine and the mechanism of carboxyl transfer by carboxylbiotin in acetyl CoA carboxylase. The possibility that idiosyncratic appearance of a wide range of acyl substituents in myelin basic protein could be related to a peculiar involvement of ribosomal pseudouridine is mentioned.

Almost 40 years ago, Spector and Keller (3) reported that the N-1 position in uracil could transfer acyl groups into peptide linkage (3), but the discovery was made at a time when RNA was thought to contain only the four classical nucleosides. The authors noted that the N-1 position of uracil is blocked in the corresponding classical nucleoside (uridine) of RNA and concluded that the property was of chemical, not of biochemical interest. Ironically, a "fifth nucleoside" had been isolated from RNA (4) a year before the Spector and Keller report, but 3 years passed before the fifth nucleoside, the first modified nucleoside found in RNA, was identified as a uridine isomer, pseudouridine (5–7).

The possible relevance of the Spector and Keller finding for a prominent role of pseudouridine during peptidyltransfer in the ribosome was only articulated for publication during the past few years (8, 9). In this brief time, the Ofengand laboratory has shown that all pseudouridine residues in the RNA of large ribosomal subunits from Escherichia coli (9ψ residues) through human (54 ψ residues) are present in the peptidyl transfer region of the ribosome (10–13). Where it was once possible to envision a network of transfer sites in the ribosome, from tRNA termini via rRNA termini into peptide linkage, it is now possible to envision a network of ψ sites.

The ψ sites are not evolutionarily conserved in the primary and secondary structure of rRNA but could possibly constitute a network of transfer sites within the stereostructure of the ribosome. Two residues, including ψ2580 (E. coli numbering), found in most but not all (13) prokaryotic and mitochondrial large subunit RNAs so far analyzed, and ψ2594, in the "universal" Um-Gm-ψ sequence of all eukaryotic/mitochondrial large subunit RNAs so far studied, are quasi-conserved, and as with many other large subunit RNA pseudouridine residues, they are likely proximal to the 2',3' termini of ribosomal P-site tRNAs.

Correlation between the amount (14) and sequence occurrence (Um-Gm-ψ) (15) of ψ and 2'-O-methyl nucleotides in eukaryotic (wheat) rRNA was first noted more than 30 years ago. Parallel increments (10-fold) in the two types of modification between the rRNAs of bacteria and higher eukaryotes (16, 17) was also noted, as was a potential biosynthetic relation between them (18, 19). Interrelatedness of pseudouridylation and sugar-methylation in rRNA has recently been discussed in the context of function and stereostructural modeling at the peptidyltransfer center in the ribosome (9, 12). This report focuses on the more specific consideration: a direct role of ψ in aminoacyltransfer.

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‡ The abbreviations used are: ψ, pseudouridine; Um, 2'-O-methyluridine; Gm, 2'-O-methylguanosine; AU, absorbance unit(s); MBP, myelin basic protein; HPLC, high performance liquid chromatography; EI, electron impact; CI, chemical ionization.

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From the outset, it was clear that the acyl group-transfer potential of uracil (3) is greater than that observed for the vicinal 2',3'-acyloxy termini in tRNAs (1), but it was equally clear that, if it had group-transfer properties at all, the properties of \( \psi \) might differ from those of uracil. The most acidic ionizations in uracil, uridine, and pseudouridine were 9.50, 9.25 (20), and 8.97 (21), respectively, and the very high group-transfer potential of the \(-\text{CO-NH}^{-}\) dissociation in uracil, which contains the N-1 (O-2) positions (22), was revealed by experiment (3).

As in uracil, the most acidic \(-\text{CO-NH}^{-}\) ionization in pseudouridine is allied with a very strong bathochromic shift (\(-20 \text{ m}\text{u}\)) of the UV maximum; however, the most acidic \(-\text{CO-NH}^{-}\) ionization in uridine is not allied with a bathochromic shift of its UV maximum, and it is located between two carboxyl groups (see Ref. 22). It was therefore posited (8) that N-1 (O-2) in pseudouridine, but not N-3 (O-4) in uridine, might share the high acyl transfer potential invested in N-1 (O-2) of uracil (3). An analogous situation obtains in urate/urate acid. For example, in urate, N-3 (analogous to N-1 in uracil) is the most acidic site in the heterocycle, whereas N-1 (analogous to N-3 in uracil) is flanked by carboxyl groups and as with N-3 in uracil, is a relatively weak dissociation in the purine base (23).

It could only be determined by experiment whether \( \psi \) shares the anticipated (8) acyl group-transfer properties of uracil. It was first necessary to establish whether pseudouridine, like uracil, can accept acyl groups in its heterocycle and then whether pseudouridine can mediate transfer of the acyl substituent into peptide linkage. For this purpose, a model system involving the N-terminal sequence of human myelin basic protein was developed, and the positive findings are described in this report.

**MATERIALS AND METHODS**

Acetylation of Uracil, Uridine, and Pseudouridine—The method of Spector and Keller (3) was used to prepare 1-acetyluracil. Briefly, 1.0 g of uracil (Sigma) was refluxed at 165°C for 4 h in 10 mL of acetic anhydride. The solid that formed on cooling was recrystallized from 50% anhydride, and dried. Anhydride, and dried anhydride. The solid that formed on cooling was recrystallized from 50%

Acetylation of Peptide 1–21—The acetylation of peptide 1–21 was carried out using either 1-acetyluracil, acetylduridine, or acetyl pseudouridine. For reaction, 1.0 mg of peptide was dissolved in 1.0 or 2.0 mL of water to which was added 4–10 mg of acetylated base or nuclease. After 5 h at room temperature, the solutions were centrifuged at 12,000 \( \times \) g for 5 min, and the supernatant solutions were applied to a reverse phase HPLC column in order to separate the acetylated peptide from unreacted starting materials. The acetylated 1–21, dissolved in 0.05% trifluoroacetic acid, was purified on a Pharmacia C2/C18 Pep-S column using a 50-min linear gradient from 0 to 60% acetonitrile. Discrete fractions of material absorbing at 226 nm were analyzed by mass spectrometry.

Carboxymethylcellulose (CM52) Chromatography—Small amounts of acetylpseudouridine, which co-eluted with the acetylated peptide, were removed by carboxymethylcellulose chromatography. The CM52 column was equilibrated in 0.05 M ammonium formate buffer (pH 4.5). The sample was dissolved in the same buffer, applied to the column (0.5 × 5 cm), and washed with this buffer until the nuclease eluted. The acetylated peptide, bound to the column, was eluted with 1 M ammonium formate buffer (pH 4.5) and used for mass spectrometry.

Deacetylation of Peptide 1–21—The acetylated peptide was dissolved in a freshly prepared 0.2 M ammonium bicarbonate buffer containing 1 mM EDTA and 5 mM 2-mercaptoethanol (pH 7.7). Acylamino acid peptidase (Boehringer Mannheim) was added at one-fifth of the concentration (w/w) of peptide. The reaction mixture was incubated for 8 h at room temperature, and, after lyophilization, the residue was dissolved in water and desalted by HPLC. An aliquot was sent for mass spectrometric analysis. A second aliquot of the peptide was sequenced in a Protein 2020 gas-phase sequencer.

Electrospray Mass Spectrometry—Mass spectra were obtained on a SCIEX API-III triple quadrupole mass spectrometer (Thornhill, Ontario, Canada). All samples were dissolved in water and introduced into the mass spectrometer by flow injection with acetonitrile/water/formic acid (50:49:1) as the eluent at a flow rate of 20 \( \mu \)L/min.

EI and CI Mass Spectrometry—Electron impact (EI) and chemical ionization (CI) mass spectra were obtained using the Fison VG-Analytical mass spectrometer (Manchester, United Kingdom). The EI condition follows: electron energy 70 eV, source temperature 200°C, and 100 \( \mu \)A trap current. The mass spectrometer was calibrated using perfluoroalkane, and the resolution was set at about 800-1000. The data were collected using the VG software with a PDP-11–24 data system. The sample was introduced into the mass spectrometer by direct probe insertion, and mass spectra were obtained with a mass range between 50 and 500, continuously, while the probe was heated to 300°C. The CI conditions were as follows: electron energy 100 eV, 1 mA emission current, and methane reagent gas.

**RESULTS**

Acetylation of Uracil—Uracil was acetylated as described under “Materials and Methods.” To identify the product, acetylated uracil was subjected to EI mass spectrometry (Fig. 1). A molecular ion of m/z 154 (i.e. 42 atomic mass units (an acetyl group) greater than uracil) was obtained; no diacetylated uracil was found. The EI mass spectrum showed three predominant fragment ions: m/z 126 (loss of CO), m/z 112 (loss of ketene), and m/z 69 (loss of HNCO from the m/z 112 fragment or loss of AcNO from molecular ion). The EI mass spectrum of uracil (27) shows loss of HNCO from the molecular ion, and this elimination primarily involves loss of C-2 and N-3 through a retro-Diels-Alder mechanism, as established by Rice et al. (28). The dominant m/z 69 fragment ion indicates that the acetyl
group is located at either N-1 (loss of HNCO from the m/z 112) or N-3 (loss of AcNCO from the molecular ion) of uracil and not at O-2 or O-4, where the retro-Diels-Alder mechanism would not be possible. The data do not permit unequivocal assignment of the site of acetylation, but this was established, definitively, by x-ray structure analysis of an acetylated crystal.

Crystal Structure of Acetylated—The crystal structure determination of acetylated uracil has proved that the site of acetylation is exclusively at N-1. The molecular structure and bond lengths in the molecule are shown in Fig. 2. The uracil ring is planar, as are the acetyl group and N-1; the angle between these planes is 11°. Bond lengths and angles in the molecule have normal values; the C-5–C-6 distance (1.31 AU) is somewhat shorter, and the C-4–C-5 distance (1.45 AU) is a little longer than the corresponding lengths (1.34 and 1.43 AU) in uracil (29), possibly indicating a small inaccuracy in the position of C-4. Given the quality of the crystal, this is not remarkable. The co-ordinates are shown in Table I. The molecules in the crystal are hydrogen bonded from N-3–H to O-2 (N...O distance = 2.62 AU) in head-to-head zig-zag fashion, forming two sets of ribbons along opposite bc diagonals of the cell. The ribbons are held together through van der Waals’ interactions.

Acetylation of Uridine and Pseudouridine—Because the N-1 position in uridine is involved in N-glycosyl linkage to ribose, acetylation of the base could only occur at N-3. The EI mass spectrum of acetylated uridine is similar to that discussed by McCloskey (27) for triacetoylated uridine. The CI mass spectrum shows a protonated molecular ion at m/z 371, which confirms that only the hydroxyl groups of ribose are acetylated and that acetylation does not occur in the base of uridine. The CI mass spectrum of acetylated pseudouridine shows a prominent molecular ion at m/z 413, corresponding to the addition of four acetyl groups. Presumably three of these are in the sugar as in uridine and one is in the base. The EI mass spectrum showed m/z 352, 310, 250, 208 (base peak), 191, and 141 as the major fragment ions. The m/z 352, 310, 250, 208, and 191 are formed by elimination of either acetic acid, ketene, or acetyl groups. Owing to the strong C–C bond between uracil and ribose substituents of pseudouridine, no B + H, B + 2H or loss of HNCO was detected. Although the evidence is not discriminating, it is likely the site of base acetylation is at N-1 in the base of acetylated pseudouridine as it is in acetylated uracil (Fig. 2).

Acetylation of Peptide 1–21 of Myelin Basic Protein by Acetyl pseudouridine—A peptide consisting of the first 21 amino acids of MBP was synthesized as described earlier (26). The peptide was characterized by amino acid sequencing and mass spectrometry. The electrospray mass spectrum of peptide 1–21 shows doubly, triply, and quadruply charged protonated molecular ions, with the triply charged species most abundant. The molecular weight of 2304.5 agrees well with the theoretical value (monoisotopic mass = 2304.2; average mass = 2304.6). The peptide (300 µg) was incubated with 3 mg of acetyluracil, and the mass of the resulting acetylated peptide was 2347.2 by mass spectrometry, with a yield of approximately 60% as determined from the relative peak heights. From these data, we concluded that 1 mol of acetate was added per mol of peptide. To confirm that the acetyl group was transferred to the N terminus, the acetylated peptide was treated with acylaminopeptidase as described under “Materials and Methods.” The mass of the peptide was reduced from 2347.2 to 2304, which represents a loss of 42 mass units, in agreement with the conclusion that peptide 1–21 of MBP was acetylated at its N terminus.

Acetylation of Peptide 1–21 of Myelin Basic Protein by Acetyl pseudouridine—in separate experiments, peptide 1–21 was reacted in water (pH 6.0) with a 24-fold molar excess of acetylated uridine or acetylated pseudouridine. Each reaction mixture was fractionated on HPLC as described under “Materials and Methods.” The material collected at 26–27 min was applied to a CM52 ion-exchange column as described under “Materials and Methods.” The peptide material (confirmed by amino acid sequencing and mass spectrometry, with a yield of approximately 60% as determined from the relative peak heights. From these data, we concluded that 1 mol of acetate was added per mol of peptide. To confirm that the acetyl group was transferred to the N terminus, the acetylated peptide was treated with acylaminopeptidase as described under “Materials and Methods.” The mass of the peptide was reduced from 2347.2 to 2304, which represents a loss of 42 mass units, in agreement with the conclusion that peptide 1–21 of MBP was acetylated at its N terminus.

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and, as demonstrated here for uracil (Fig. 2), that the fourth hydroxyl functions in the ribosyl substituent of the nucleoside.

Previously shown for acetyluridine (27), that in acetylpseudouridine, contains four acetyl substituents. We have assumed, as previously, and acetylation of pseudouridine yields a product that conditions yields a product that contains three acetyl substituents. Spector and Keller (3). Acetylation of uridine under the same conditions yielded a product that contained one acetyl substituent. We have confirmed that monoacetyluracil is made during reflux in acetic anhydride, and we have characterized the product by mass spectrometry. Most significantly, x-ray crystallography has established that acetylation occurs at N-1 as predicted by Spector and Keller (3).

Using the procedure described by Spector and Keller (3), we have confirmed that monoacetyl uracil is made during reflux in acetic anhydride, and we have characterized the product by mass spectrometry. Most significantly, x-ray crystallography has established that acetylation occurs at N-1 as predicted by Spector and Keller (3). Acetylation of uridine under the same conditions yields a product that contains three acetyl substituents, and acetylation of pseudouridine yields a product that contains four acetyl substituents. We have assumed, as previously shown for acetyluridine (27), that in acetylpseudouridine, three of the acetyl substituents are at the 2′-, 3′-, and 5′- hydroxyl functions in the ribosyl substituent of the nucleoside and, as demonstrated here for uracil (Fig. 2), that the fourth acetyl substituent is at the N-1 position of uracil.

Because acetyluracil, but not acetylpseudouridine can transfer an acetyl group to the N′ terminus of peptide 1–21 of human myelin basic protein, it is assumed that N-1 acetylation in the N-heterocycle of acetylpseudouridine accounts for its similar ability to transfer an acetyl group to the N terminus in peptide 1–21 of MBP. This strengthens speculation (8) that pseudouridine in the ribosome could serve to transfer acyl groups of high group transfer potential during protein biosynthesis, possibly in addition to, or in concert with His-229 of protein L2 (30, 31).

As recently noted (9), the acyl-transfer site (N-1) in pseudouridine might participate in other ways in the catalysis of ribosomal peptidyltransfer, e.g. by proton abstraction. Whatever the case, all evidence adduced (10–13) during the 3 years since publication of the ψ hypothesis (8) has only amplified the notion that pseudouridine, in one capacity (8) or another (9), has a central, significant, and previously unheralded role in the activity of the peptidyl transfer center of the ribosome.

By contrast, the possibility that a circuit of vicinal hydroxyl groups might be engaged in transferring activated aminoacyl residues from the 2′,3′ termini of tRNA molecules into peptide linkage, which had once seemed plausible when 5, 5.8, 18, and 26 S rRNAs were shown to have 2′,3′-hydroxyl termini (15, 32–35), declined steadily over the years. Accumulated information gradually revealed that the 2′,3′ termini of rRNA molecules are not located in the peptidyl transfer center of the E. coli ribosome. In the first instance, it was shown that one of the Shine-Dalgarno hypotheses (36) was correct in placing the 3′-terminus of E. coli 16 S rRNA in the immediate vicinity of the (mRNA) decoding site (37), not the peptidyltransfer site of the ribosome.

Although an explanation of the reactivity of the N-1 position in uracil is lacking, the ureido structure assumes first importance, as it does in all putative functions for pseudouridine (9). We suggest that a mechanism similar to that proposed for transfer of carboxyl groups of high group-transfer potential by biotin may be operative, also, in the transfer of acyl groups of high group-transfer potential by pseudouridine. The ureido group in biotin is the catalytically active part of the acetyl-CoA carboxylase molecule to which CO2 of high group-transfer potential has been added (38, 39).

Crystallographic studies suggest that the reactivity of N-1′ in biotin results in a partial enolate at O-2′, which is in turn responsible for deprotonation of N-1′, rendering this site nucleophilic and able to accept CO2 of high group-transfer potential, i.e. N-1′ can thereby accept the active CO2 that is generated in an ATP-dependent reaction. It seems plausible that reactivity of the N-1 position in uracil (pseudouridine) is likewise the result of partial enolate formation at the exocyclic O-2 (see Ref. 22). Another similarity, this time with respect to the subsequent mechanism of transfer, is noteworthy. Carboxyltransfer (via biotin) between donor and acceptor in acetyl-CoA carboxylase occurs by a gauche-trans rotation about the 2–6 and 6–7 bonds in the valeryl side chain of biotin (38), and this is reminiscent of the notion that increased rotational flexibility about the C–C glycosyl bond in pseudouridine, relative to an N–C glycosyl bond in uridine, may be a significant consideration in transfer of aminoacyl groups (via pseudouridine) between P and A sites in the ribosome (see Ref. 9).

The (1–21) N-terminal peptide of MBP was chosen as a model peptide for these studies because its acylation by a wide variety of fatty acids (40) may be indicative of an idiosyncratic pathway of N-acylation in MBP. It seems that the ribosome itself, via pseudouridine residues in the peptidyl transfer region, might serve to transfer acyl groups to N′ termini in MBP. For instance, N′ acetylation occurs after about 25 amino acids are added to the growing polypeptide (see Ref. 41), and a recent study of the routing of N′ termini through the E. coli ribosome shows the exit channel is wide enough to allow a nascent polypeptide to fold; residue 25 in a nascent polypeptide can cross-link to residue 2585 in 23 S RNA (42), which neighbours ψ3280 in the peptidyl transfer region.
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