ENZYMATIC DEMODIFICATION OF TRANSFER RNA SPECIES CONTAINING N^6-(Δ^2-ISOPENTENYL)ADENOSINE.

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Summary: An enzyme activity has been detected in bacterial cell extracts and liver homogenates which removes the isopentenyl side chain from isopentenylated species of transfer RNA. The site of cleavage does not involve the allylic double bond in the side chain but seems to involve C-N bond cleavage. The enzyme shows no activity when the free nucleoside is used as a substrate. The presence of this enzyme suggests that cells may have the ability *in vivo* to demodify species of tRNA which contain isopentenyl adenosine nucleotides.

INTRODUCTION

Species of aminoacyl-transfer RNA which respond to codons beginning with the letter U often contain the modified nucleoside N^6-(Δ^2-isopentenyl)adenosine (i^6A) or 2-methylthio-i^6A adjacent to the 3' end of the anticodon (1,2). The extent of modification, that is the formation of i^6A or 2-methylthio-i^6A, appears to vary with the growth conditions of the cells under investigation. The presence of i^6A or its methylthio derivative seems to be universal in so far as they occur in tRNA species from most organisms including plants, mammals and microorganisms (3). Although the exact explanation for the regular occurrence of these modified nucleosides adjacent to the anticodon is not known it is clear that the presence and integrity of such modifications is necessary for proper codon-anticodon interaction.

In this paper we report the existence of an enzyme activity which removes the isopentenyl moiety from i^6A residues at the trans-

fer RNA level. The presence of this enzyme supports the view that the cell may use a facile mechanism for regulating the availability of certain species of transfer RNA - namely modification (isopentenylation) and demodification (removal of isopentenyl groups) at the macromolecular level.

EXPERIMENTAL

Materials. Quantities of 2-[^{14}C]-DL-mevalonate (10.3 mc/mm) and 8-[^{14}C]-adenine (51.1 mc/mm) were obtained from Amersham. 5-[^{14}C]-DL-mevalonate (11.8 mc/mm) was purchased from Schwarz-Mann and 2-[^{3}H]-DL-mevalonate (6.25 c/mm) was obtained from New England Nuclear. Lyzozyme and ribonuclease A preparations were purchased from Sigma Chemicals. All other chemicals were commercial preparations.

Labelling and Isolation of transfer RNA.

Lactobacillus acidophilus cells (ATCC 4963) were grown in the regular strength semisynthetic medium described by Thorne and Kodicek (4), supplemented with 0.68 µmoles of 2-[^{14}C]-DL-mevalonic acid per liter of culture medium. After the cultures had grown to late log phase (about 6 hours) the cells were harvested by centrifugation. Transfer RNA was isolated and purified according to the phenol extraction procedure of Holley (5) modified as follows. The DEAE-cellulose column was washed with ten bed volumes of 0.1M NaCl in 0.1M Tris-chloride, pH 7.6. The transfer RNA was eluted with 1.0M NaCl in the same buffer. The RNA solution was desalted by dialysis or by ultrafiltration on UM-10 membranes (Amicon Corporation). The resulting transfer RNA has a sedimentation value of 4.12. The specific activity of a typical preparation was 1090 dpm per A_{257} unit of transfer RNA.

The position of the label in the transfer RNA was verified by chemical and enzymic hydrolysis. Alkaline hydrolysis of transfer RNA labelled by the method described gave a product with R_f of 0.49 in a solvent system consisting of isopropyl alcohol, concentrated ammonium hydroxide and water (7:1:2). Cochromatography with i^6Ap confirmed the identity of the expected product. Acid hydrolysis of the nucleoside obtained from i^6A monophosphate after phosphomonoesterase treatment gave the two expected products (6). In all transfer RNA preparations labelled with 2-[^{3}H]-mevalonate or 2-[^{14}C]-mevalonate the label is confined to i^6A residues in the intact transfer RNA molecules (1).

Preparation of the Enzyme Extract.

Liquid cultures of L. acidophilus (ATCC 4963) were grown in the semisynthetic medium of Thorne and Kodicek (4) and the cells were harvested by continuous flow centrifugation. The cells were washed with 0.01M Tris-chloride pH 7.5 containing 0.001M $MgCl_2$ and 0.001M mercaptoethanol. The final pellet was suspended in the above buffer using 0.5 ml per gram of cells. The cells were lyzed by incubating the suspension with lyzozyme (10 mg/gm cells) for one hour at 37°. The digest was applied to a sephadex G-75 column (40 x 2 cms) and the column eluted with the buffer described above. Fractions containing active enzyme were pooled.

A working solution of enzyme was prepared from fresh bovine liver as follows. The liver sample was homogenized in two volumes

of 0.1M Tris-chloride pH 7.6 buffer containing 0.01 $MgCl_2$. The homogenate was filtered through several layers of cheesecloth to remove buoyant material. The filtrate was centrifuged at 42,000 x g for 45'. The supernatant solution contained active enzyme and served as a working solution.

Enzyme Assays. Protein determinations were made according to the method of Lowry et al. The amount of enzyme activity was measured by determining the amount of radioactivity released from the substrate transfer RNA. A typical incubation mixture contained 0.5 to 1.5 A_{257} units of labelled transfer RNA, 50-75 micrograms of protein and 75 μmoles of Tris-chloride buffer, pH 7.6 in a final volume of 0.2 mls.

After incubation at 37° for one hour the reaction mixture was spotted directly onto Whatman #3 MM chromatography paper. After development in a solvent system consisting of isopropyl alcohol:concentrated ammonium hydroxide:water (7:1:2), the chromatogram was cut into 0.5 cm segments and the radioactivity in each segment measured in a scintillation counter.

DISCUSSION AND RESULTS

The relationship between the specific activity of transfer RNA and the concentration of 2-[^{14}C]-mevalonate in the culture medium is shown in Fig. 1.

For these studies it is of paramount importance to ensure that all of the radioactivity in the substrate transfer RNA is con-

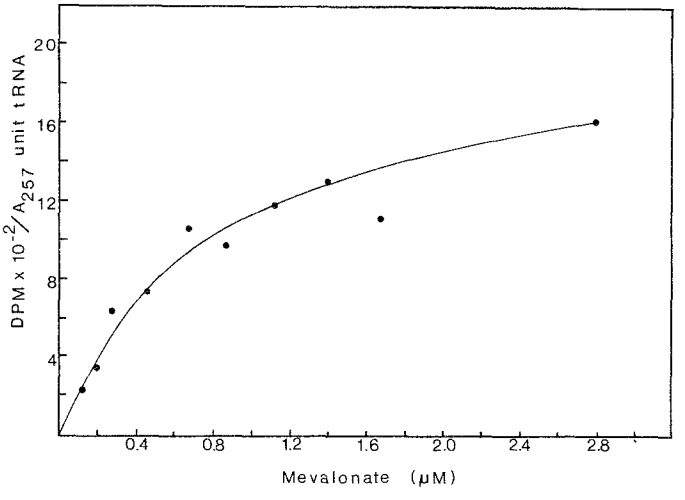

Fig. 1. The specific activity of transfer RNA in response to varying 2-[^{14}C]-DL-mevalonate concentration. The growth conditions and tRNA purification were as described in the text.

fined exclusively to isopentenyl adenosine residues. Each preparation of ^{14}C- or ^{3}H-labelled tRNA is chromatographed (see methods) before and after hydrolysis with 1M naOH. Intact transfer RNA remains at or near the origin (R_f=0.04). The mononucleotide i^6Ap (2' and 3' isomers co-chromatograph) is the only labelled product (R_f=0.49) obtained by alkaline hydrolysis. After phosphomonoesterase treatment of the labelled nucleotide, all of the radioactivity is recovered as the nucleoside, i^6A. These experiments prove that the substrate transfer RNA employed in these experiments carries the radioactivity from labelled mevalonate exclusively in the side chains of isopentenyl adenosine residues.

When the side-chain-labelled transfer RNA is incubated with enzyme the radioactivity is lost from the transfer RNA and migrates as a fast moving fragment near the solvent front (Table 1). The activity was destroyed when the enzyme solution was heated to 100° for 10 minutes. To determine that the radioactivity was released by cleavage of the isopentenyl side chain and not as a consequence of possible nuclease action on the transfer RNA, the following experiment was performed. Doubly-labelled i^6A-tRNA was isolated from

TABLE 1. Enzymic hydrolysis of isopentenyl-labelled i^6A-tRNA[a]

Assay	Substrate dpm	Product dpm
i^6A-tRNA	1515 ± 12	0
i^6A-tRNA + enzyme	36 ± 14	1445 ± 16

[a] The isopentenyl side chain contains ^{14}C label in the terminal methyl groups. Assay values are means ± S.E.M. of eight determinations.

L. acidophilus cells which had been grown in the presence of 8-[^{14}C]-adenine and 2-[^{3}H]-mevalonate. Under incubation conditions in which 78 to 95% of the ^{3}H counts were removed from this doubly labelled i^{6}A-tRNA there was no disappearance of ^{14}C counts from the substrate tRNA (see Table 2). This result indicates that there is no detectable hydrolysis of the tRNA by nuclease action and supports the notion that the enzyme is removing the isopentenyl side chain from i^{6}A residues in the substrate i^{6}A-tRNA.

From a chemical viewpoint one might expect the cleavage of the isopentenyl side chain to occur at the allylic double bond in the side chain or at the C-N bond linking the side chain to the exocyclic amino nitrogen of adenylate residues. When 2-[^{14}C]-mevalonate is used as a precursor the isopentenyl side chain is labelled at the terminal methyl groups. When 5-[^{14}C] mevalonate is used as a precursor the isopentenyl adenylate residues in i^{6}A-tRNA are labelled at the carbon atom adjacent to the amino nitrogen on carbon 6 of adenosine. Thus, the use of i^{6}A-tRNA biosynthesized using each

TABLE 2. Enzymic hydrolysis of i^{6}A-tRNA which is labelled on both the purine ring and the isopentenyl side chain.

Assay	Substrate (dpm) ^{3}H	Substrate (dpm) ^{14}C	Product (dpm) ^{3}H	Product (dpm) ^{14}C
i^{6}A-tRNA[a]	8805	1778	-	-
i^{6}A-tRNA + enzyme	1850	1590	6470	-
i^{6}A-tRNA + enzyme[b]	424	1545	7987	-

[a] The ^{3}H label is on the methyl carbons of the isopentenyl side chain and the ^{14}C label is carbon atom 8 of adenine.

[b] Incubation time was extended to four hours.

TABLE 3. Enzymic hydrolysis of i^6A-tRNA biosynthesized from 5-[^{14}C]-mevalonate and 2-[^{14}C]-mevalonate.

tRNA	Substrate dpm	Product dpm	% hydrolysis
i^6A-tRNA (from 5-[^{14}C]-mevalonate	810	641	79
i^6A-tRNA (from 2-[^{14}C]-mevalonate	758	582	77

of these precursors separately permits one to distinguish between allylic double bond cleavage and C-N bond cleavage. The results of such an experiment are shown in Table 3. The two types of i^6A-tRNA were hydrolyzed to the same extent under all conditions tested. That is, the extent of removal of carbon counts from the proximal and distal ends of the isopentenyl side chain was identical. This shows that the cleavage point is not the allylic double bond on the isopentenyl side chain and suggests that the catalysis involves C-N bond cleavage.

The presence of this enzyme suggests that cells may have the ability *in vivo* to demodify i^6A-containing species of transfer RNA. Others have shown that chemical alteration (7) or incomplete biosynthesis (8) of i^6A residues leads to an altered codon response or a dimunition of transfer RNA function. White *et al.* (9) have demonstrated a modification in Drosophila transfer RNA involving the nucleotide Qδp and Schaeffer *et al.* (10) have described the existence of ribothymidine- and pseudouridine-forming activities in *E. coli*. It remains to be shown whether these modifying enzymes or the demodifying activity described in the present paper play any role in the regulation of protein synthesis at the translational level.

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REFERENCES

1. Peterkofsky, A., and Jesensky, C. (1969) Biochem., 8, 3798-3809.
2. Yamada, Y., Nishimura, S., and Ishikura, H. (1971) Biochim. Biophys. Acta, 247, 170-174.
3. Hall, R. H. (1970) in Progress in Nucleic Acid Research and Molecular Biology (J. N. Davidson and W. E. Cohn eds.) Vol. 10 Academic Press, New York. pp. 57-86.
4. Thorne, K. J., and Kodicek, E. (1962) Biochim. Biophys. Acta 59, 273-279.
5. Holley, R. W. (1963) Biochem. Biophys. Res. Comm. 10, 186-188.
6. Robins, M. J., Hall, R. H. and Thedford R. (1967) Biochemistry 6, 1837-1848.
7. Fittler, F. and Hall, R. H. (1966) Biochem. Biophys. Res. Comm. 25, 441-446.
8. Gefter, M. L. and Russell, R. L. (1969) J. Mol. Biol. 39, 145-157.
9. White, B. N., Tener, G. M., Holden, J. and Suzuki, D. T. (1973 J. Mol. Biol. 74, 635-651.
10. Schaefer, K. P., Altman, S. and S. and Söll, D. (1973) Proc. Nat. Acad. Sci. U.S.A. 70, 3626-3630.