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SYNTHESIS OF N^4-SUBSTITUTED CTP BY MAMMALIAN CTP SYNTHETASE

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Highly purified CTP synthetase from Ehrlich ascites tumor cells catalyzes the formation of N^4-substituted CTP from UTP and hydroxylamine and its derivatives. The products with hydroxylamine and O-methylhydroxylamine were identified as N^4-hydroxyCTP and N^4-methoxyCTP by absorption spectra and chromatographic behavior on Dowex column, respectively. The weak nucleophilic amines such as methylamine, ethylamine or diethylamine and a less nucleophilic amine, sulfamic acid, did not react with UTP. These results suggest that the nucleophilicity and basicity of amines are important in the enzymic reaction with UTP.

Hydroxylamine and hydrazine are mutagens classified as simple non-alkylating agents (1). When these compounds affect extracellular viruses or cells which do not metabolize these amines, certain residues in the genomes may be modified by direct chemical reaction between amines and the components of nucleic acids. Whereas, in metabolizing cells, the nucleotides which are chemically or enzymatically modified by these amines may be incorporated into nucleic acids (1-3). Bacterial CTP synthetase converts UTP to N^4-hydroxyCTP in the presence of hydroxylamine (4) and N^4-hydroxycytidine residues are incorporated into RNA by RNA polymerase (5,6). The present study shows that in the presence of hydroxylamine or its derivatives mammalian CTP synthetase catalyzes the formation of N^4-substituted CTP.

MATERIALS AND METHODS

CTP synthetase was highly purified from Ehrlich ascites tumor cells as previously reported (7). The reaction with amines was carried out under the same conditions as those of CTP synthesis

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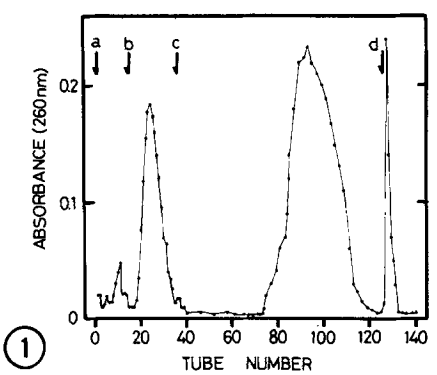
with ammonia using [^{14}C]UTP (7) except that 100 mM hydroxylamine or hydrazine was used instead of ammonia. The final pH of the reaction mixture was 7.4. After chromatography of the reaction mixture on polyethyleneimine cellulose plate in 0.8 M ammonium sulfate solution at 4°C (8) or in 0.5 N HCl at room temperature, the plate was dried and exposed to X-ray film for 2 overnight. The spots of the products were identified by matching the plate with developed X-ray film, excised and counted. N^4-hydroxyCTP and N^4-methoxyCTP were chemically synthesized by the methods of Budowsky et al (9) and purified by anion exchange chromatography using Dowex 1 X 8 column.

RESULTS

Reaction with hydroxylamine: The reaction product with UTP and hydroxylamine was eluted after UTP with 0.1 N HCl containing 0.1 M KCl by anion exchange chromatography (Fig. 1). CTP which was eluted before UTP with 0.01 N HCl containing 0.1 M KCl was not detected. Absorption spectra of this new product shown in Fig. 2



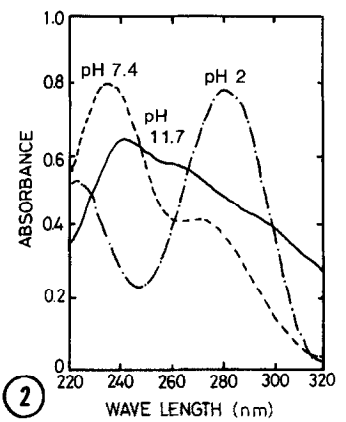


Fig. 1. Anion exchange chromatography of the reaction products with hydroxylamine.
The reaction was carried out for 30 min and terminated by boiling. Clear supernatant obtained by centrifugation was diluted with 0.01 N HCl and applied on Dowex 1 X 8 column (Cl form, 1.4 x 6 cm). The products were eluted in order; a. 0.01 N HCl, b. 0.01 N HCl + 0.05 M KCl, c. 0.01 N HCl + 0.1 M KCl, d. 0.1 N HCl + 0.1 M KCl.
ADP and ATP were eluted with a and b, respectively. UTP was eluted with c. CTP was eluted immediately with c before UTP. New product was eluted immediately with d.

Fig. 2. Absorption spectra of the reaction product with hydroxylamine.
Fraction eluted with d (Fig. 1) was concentrated in vacuo and desalted with Sephadex G10 column. Spectra were measured at 3 different pH, 2.0, 7.4 and 11.7.

was identical to those of chemically synthesized N^4-hydroxyCTP and similar to those of N^4-hydroxyCMP reported (9). The product from [^{14}C] UTP was one and located between CTP and ATP on the autoradiogram developed in 0.8 M ammonium sulfate solution. Rf value of the product was identical to that of N^4-hydroxyCTP chemically synthesized. This product yielded faint blue color with $FeCl_3$ reagent (10). Thus the product with hydroxylamine was tentatively identified as N^4-hydroxyCTP. The maximum activity for N^4-hydroxyCTP synthesis was observed in the pH ranges between 7.2 and 8.0, whereas the optimum pH for CTP synthesis with glutamine or ammonia was 8.6 (11).

Requirements and Stoichiometry: The formation of N^4-hydroxyCTP depended absolutely on the presence of enzyme, Mg^{2+}, UTP and ATP. GTP which is an essential activator for the glutamine reaction (7) was not necessary in the presence of ATP. GTP could replace ATP as high energy donor like in CTP synthesis (7), but the activity with 10mM GTP was about 15% of that with ATP. When UDP, UMP or uridine was used instead of UTP, product formation was not observed. Cytidine and cytidine nucleotides also did not react with hydroxylamine under the conditions used. Stoichiometry of the reaction was examined as previously reported (7). To the amount of N^4-hydroxyCTP formed, equivalent amount of ADP from ATP was produced. These results indicate that N^4-hydroxyCTP was synthesized by the enzymic reaction catalyzed by CTP synthetase and not by direct chemical reactions.

Reaction with other amines: Reactivity with hydroxylamine, its derivatives and hydrazine was summarized in Table I. All the reaction with these amines depended on the presence of UTP, ATP, Mg^{2+}, and enzyme. Optimal pH of the reaction was observed between 7.2 and 8.0. We could not separate the products with these amines except N^4-hydroxyCTP from CTP by thin layer

TABLE I REACTION WITH AMINES

Amines	Activity (μmol/hr/mg protein)
NH_3	6.0
NH_2OH	15.7
NH_2OCH_3	3.3
CH_3NHOH	2.7
NH_2NH_2	4.8

chromatography either in 0.8 M ammonium sulfate solution or in 0.5 N HCl because of the same Rf values of these products as that of CTP. The reaction product with O-methylhydroxylamine was eluted at the position of N^4-hydroxyCTP on Dowex column chromatography and was tentatively identified as N^4-methoxyCTP by its absorption spectra (6).

The activity with hydroxylamine was highest among the amines examined, but the apparent Km value for hydroxylamine was about 16 mM which was higher than that for ammonia (1 mM) (11).

Preliminary experiments showed that CTP synthetase partially purified from mouse liver could also catalyze the formation of N^4-substituted CTP in the presence of hydroxylamine, o-methylhydroxylamine and hydrazine. Thus the enzymes from normal and malignant tissues can catalyze the reaction with UTP and amines. The amines which react with UTP in the presence of the enzyme are thought to be strong and moderate nucleophilic derivatives of ammonia. Indeed, weak nucleophilic derivatives such as methylamine, ethylamine, or diethylamine, and a less nucleophilic derivative, sulfamic acild, showed no reactivity with UTP. Mono-, di- and triethanolamine, of which pKa values are 9.0, 8.88, 7.77, respectively (12), did not react with UTP. These results suggest that nucleophilicity, basicity and steric properties of the amines are important in the enzymatic reaction with UTP.

DISCUSSION

Hydroxylamine and hydrazine are nucleophilic derivatives of ammonia and may act as amido donor in the amidotransferase reactions involved in nucleotide biosyntheses since ammonia can replace glutamine as amido donor in these reaction. In the presence of hydroxylamine, AMP synthetase converts IMP to N^6-hydroxyadenosine nucleotide (13) and XMP aminase converts IMP to N^2-hydroxyGMP which is a powerful inhibitor of this enzyme (14). Formylglycinamide ribotide amidotransferase (15) and phosphoribosylamidotransferase (16) also react with hydroxylamine or hydrazine. The nucleotides modified by these amines may be substrates or substrate-like inhibitors in the processes of nucleic acid biosynthesis. The present studies showed that mammalian CTP synthetase could catalyze the formation of N^4-substituted CTP. N^4-substituted CTP is a direct precursor for RNA synthesis (5.6) and N^4-substituted deoxyCTP is incorporated into DNA inducing a tautomeric shift in bacterial systems (17). The precise mechanism of mutagenesis has not been defined in any mammalian system, but these amines induce chromosomal aberration, mitotic crossing-over, or various nuclear anomalies in mammalian cells (2,3). It is uncertain whether the reaction with amines by CTP synthetase is involved in these biological expressions, rather remains to be studied.

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