Purification and Kinetic Properties of *Neurospora* Transfer Ribonucleic Acid Nucleotidyltransferase†

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ABSTRACT: The tRNA nucleotidyltransferase of *Neurospora* has been purified 500-fold. The purified preparation catalyzed the incorporation of 24,100 nmol of AMP/hr per mg of protein at 35°. The ratio of the specific activities in the AMP and the CMP incorporation assays remained constant throughout the purification. The enzyme incorporated CMP into snake venom phosphodiesterase-treated tRNA from *Neurospora*, *Escherichia coli*, or yeast. AMP was readily incorporated into untreated yeast tRNA. No incorporation of nucleotides into DNA or rRNA was observed with ATP, CTP, UTP, or GTP. Under standard conditions, neither GMP nor UMP were incorporated into tRNA. CTP and ATP competitively inhibited the incorporation of AMP and CMP, respectively. The K_m and K_i values for each nucleoside triphosphate were very similar, suggesting the identity of the catalytic and inhibitory site(s).

The tRNA nucleotidyltransferase, originally reported by Hecht *et al.* (1958), has been isolated from different sources (for references, see Discussion). Under appropriate conditions, the enzyme can synthesize the 3′-terminal sequence of tRNAs, according to

$$\text{tRNApN}^1 + 2\text{CTP} + \text{ATP} \overset{(Mg^{2+})}{\rightleftharpoons} \text{tRNApCpCpA} + 3\text{PP}_i$$

The physiological role originally assigned to this enzyme was the *de novo* synthesis of the -pCpCpA end. The recent demonstration (Altman and Smith, 1971) that the -pCpCpA sequence is transcribed in a su_{III}^+ $tRNA^{Tyr}$ precursor suggests that the tRNA nucleotidyltransferase may have a *repair* biosynthetic function. *In vivo*, the terminal AMP is turned over at a considerable rate (Rosset and Monier, 1965); since an intact 3′ end is required for amino acid acceptance, the tRNA nucleotidyltransferase could play an important role in the synthesis of protein and its control.

In this paper, we describe a convenient procedure for the purification of tRNA nucleotidyltransferase from *Neurospora* extracts. A study of the catalytic properties of the enzyme indicated that the nucleotide substrate specificity and several kinetic parameters and properties of the *Neurospora* tRNA nucleotidyltransferase were quite different from those reported for the enzyme from other sources.

Materials and Methods

Strain and Growth Conditions. The wild-type strain of *Neurospora crassa* 74A was kindly provided by the Fungal Genetics Stock Center, Humboldt College, Arcata, Calif. Cultures were grown in 5-gal. carboys containing 10 l. of medium N (Vogel, 1956) with 2% sucrose. Each carboy was inoculated with 5×10^9 conidia and incubated at 23 ± 2° for 36–42 hr under forced aeration. The mycelia were harvested by filtration on a Büchner funnel, washed with 0.1 mM EDTA, lyophilized, and stored in evacuated desiccators.

Chemicals. [^{3}H]ATP, [^{3}H]CTP, [^{3}H]UTP, and [^{3}H]GTP were from New England Nuclear. Nonradioactive nucleoside triphosphates of the highest purity available were purchased from Sigma Chemical Co. or Calbiochem. *Escherichia coli* alkaline phosphatase and α,β-methyleneadenosine triphosphate were from Miles Laboratories. Yeast rRNA was a product of Calbiochem. Calf thymus DNA and snake venom phosphodiesterase were products of Worthington Biochemical Corporation. Bz-DEAE-cellulose and enzyme grade ammonium sulfate were from Schwarz/Mann. Sephadex G-100 and Dextran Blue were obtained from Pharmacia. Hydroxylapatite (Bio-Gel HTP) was purchased from Bio-Rad.

tRNAs. E. coli tRNA was isolated from late-log *E. coli B* cells (General Biochemicals) following a standard procedure (Zubay, 1962). *Neurospora* tRNA was isolated by a modification (Nazario, 1972) of the isopropyl alcohol fractionation of Zubay (1962). Baker's yeast tRNA was either isolated according to Holley (1967) or purchased from Calbiochem. All three tRNAs were purified further by chromatography on Bz-DEAE-cellulose (Gillam *et al.*, 1967). Untreated yeast tRNA accepted AMP readily, while CMP incorporation required digestion of the tRNA with snake venom phosphodiesterase. The phosphodiesterase treatment was carried out under the conditions of Zubay and Takanami (1964); after incubation for 20 hr at 5°, the tRNAs were reisolated by treatment with phenol (redistilled), precipitation with ethanol, and filtration through 0.45 μ Millipore disks.

The concentration of tRNA was calculated assuming an $E_{260}^{0.1\%}$ of 20 (in 0.01 N KOH).

Purification of tRNA Nucleotidyltransferase. All steps were carried out at 2–5°. Powdered mycelium (45 g) were suspended in 1 l. of 0.03 M Tris–acetate (pH 8.0) made 10 mM in mercaptoethanol and 0.1 mM EDTA. After stirring for 30 min, the suspension was centrifuged at 15,000*g* for 40 min. To the extract (fraction I) 273 g of ammonium sulfate was added with constant stirring and, after 1 hr, the precipitate was collected by

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[1] Abbreviations used are: tRNA or tRNApCpCpA for tRNA with intact 3′ termini; tRNApCpC for tRNA lacking the 3′-terminal AMP; tRNApC for tRNA lacking the terminal AMP and one CMP residue; and tRNApN for tRNA in which the complete -pCpCpA terminus has been removed.

TABLE I: Purification of tRNA Nucleotidyltransferase.[a]

Fraction	Total Vol (ml)	Protein (mg/ml)	CMP Incorporation Assay		AMP Incorporation Assay		
			Sp Act.[b]	Purifcn, -fold	Sp Act.[b]	Purifcn, -fold	Recov (%)
I Extract	880	9.7	19.2	1	45.3	1	(100)
II Dialyzed ammonium sulfate	82	18.6	170	8.8	379	8.3	150
III DEAE-cellulose pool	45	3.88	1,130	59	2,630	58	119
IV Hydroxylapatite pool	34	0.41	9,750	510	24,100	530	87

[a] The tabulated figures correspond to a preparation obtained from 45 g of lyophilized mycelium. Yeast tRNA and phosphodiesterase-treated *Neurospora* tRNA were used for the AMP and CMP incorporation assays, respectively. [b] Given in nmol/hr per mg of protein.

centrifugation; to the supernatant, 141 g of ammonium sulfate was added. The resulting precipitate was dissolved in a small volume of 0.04 M potassium phosphate (pH 7.0)–0.01 M mercaptoethanol and dialyzed for 24 hr against the same (fraction II). This fraction was applied to a 2.5 × 30 cm column of DEAE-cellulose. The column was equilibrated and washed with the dialysis buffer. The tRNA nucleotidyltransferase was eluted with a linear gradient of KCl (1000 ml, 0–0.75 M) dissolved in the same buffer. The active fractions were pooled and dialyzed overnight against 0.02 M potassium phosphate (pH 7.0), 0.1 mM Na_2EDTA, and 20% glycerol (fraction III). The sample was applied to a 2.5 × 12 cm column of hydroxylapatite equilibrated with the dialysis buffer. A linear potassium phosphate (pH 7.0) gradient (500 ml, 0.02–0.12 M) was used to elute the enzyme. The active fractions were pooled and dialyzed against 10 mM potassium phosphate (pH 7.0), 1 mM reduced glutathione, and 55% glycerol. The enzyme was stored at −20° and was essentially stable for over 1 year.

Enzyme Assays. Initial rates were measured. The standard reaction mixture contained in 0.25 ml: glycine–NaOH (pH 9.1), 30 μmol; $MgCl_2$, 5 μmol; [^{3}H]ATP (specific activity 5 Ci/mol), 0.5 μmol; yeast tRNA, 13–15 nmol; and 5–18 units of enzyme. The reaction was started by the addition of labeled ATP; after 5 min at 35°, the reaction was stopped with 3 ml of cold 10% trichloroacetic acid; the samples were mixed and filtered through 2.4 cm Whatman GF/C glass fiber circles. The filters were washed five times with 3-ml portions of 5% trichloroacetic acid, transferred to vials, and dried at 80°; before counting each vial received 10 ml of toluene-based scintillation fluid.

Unless stated otherwise, the enzyme was assayed for AMP incorporation in the absence of CTP and using untreated baker's yeast tRNA. In CMP incorporation assays, the labeled ATP was replaced by 0.125 μmol of [^{3}H]CTP (specific activity 5 Ci/mol) and the tRNA substrate was snake venom phosphodiesterase-treated tRNA. K_m values were calculated from Lineweaver–Burk (1934) plots. Except for Mg^{2+} (see Results) all double-reciprocal plots were linear. One enzyme unit catalyzes the incorporation of 1 nmol of nucleotide/hr. Specific activities are units per milligram of protein. Protein was determined according to Lowry *et al.* (1951).

Results

Purification of tRNA Nucleotidyltransferase. Table I summarizes the results obtained following the procedure described in Materials and Methods. The hydroxylapatite chromatographic profile is shown in Figure 1. The specific activity of fraction IV was 21,400 (AMP incorporation). A 530-fold purification was obtained with an overall recovery of 87%. Copurification of the AMP and CMP incorporating activities (Table I) indicated that the same enzyme catalyzed the incorporation of both nucleotides, as has been observed for the tRNA nucleotidyltransferase from other sources (Preiss *et al.*, 1961; Daniel and Littauer, 1963; Deutscher, 1970).

Molecular Weight Estimation. The molecular weight was estimated by gel filtration (Andrews, 1964) using a 2.5 × 40 cm column of Sephadex G-100 (fine). Myoglobin, bovine serum albumin, alkaline phosphatase, and Blue Dextran were used as markers. The column was equilibrated and eluted with 0.05 M potassium phosphate buffer (pH 7.0) made 0.1 mM in EDTA and 0.1 M in KCl. Fractions of 1.8 ml were collected every 10 min. The tRNA nucleotidyltransferase eluted immediately after the bovine serum albumin and its molecular weight was estimated to be 62,000.

Requirements and Specificity. The enzyme had an absolute requirement of Mg^{2+} for activity. The K_m was 5 mM. Maximal activity was observed at 15–20 mM $MgCl_2$. Higher concentra-

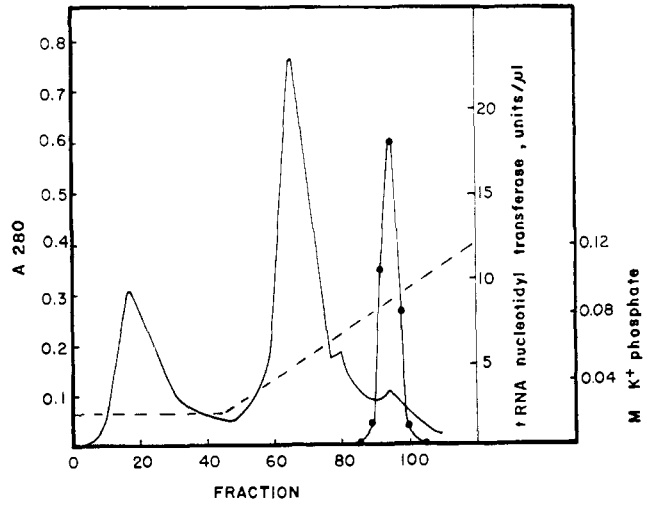

FIGURE 1: Hydroxylapatite chromatography of enzyme fraction III. Full circles, tRNA nucleotidyltransferase; the full line represents A_{280}; the broken line indicates the concentration of potassium phosphate in the eluent.

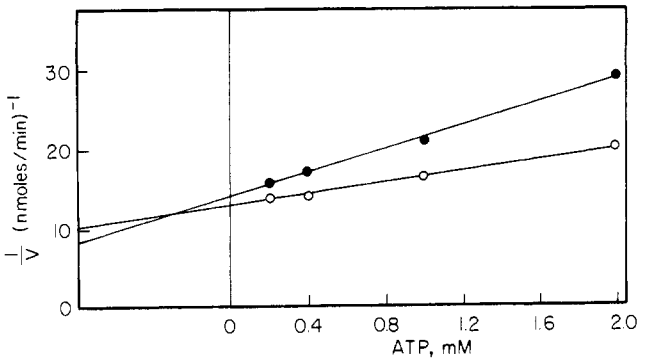

FIGURE 2: Inhibition of CMP incorporation by ATP. The concentrations of [^{3}H]CTP were either 0.2 mM (full circles) or 0.4 mM (open circles).

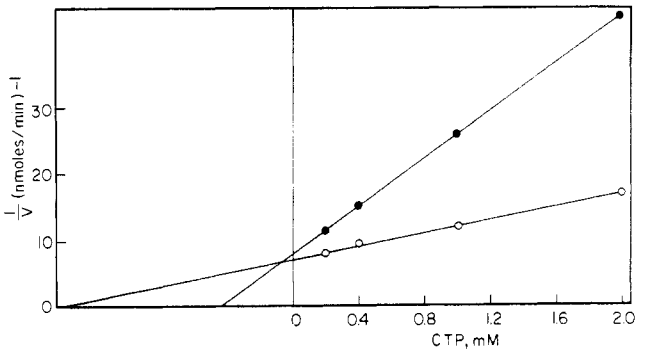

FIGURE 3: Inhibition of AMP incorporation by CTP. The concentrations of [^{3}H]ATP were either 1 mM (full circles) or 4 mM (open circles).

tions were inhibitory (81 and 45% of maximal activity at 40 and 80 mM, respectively.

The specificity of the enzyme for nucleoside triphosphates and nucleic acids is illustrated in Table II. No incorporation of AMP or CMP was observed in the presence of rRNA or calf thymus DNA. The relative rates of AMP and CMP incorporation varied, as expected, with the tRNA substrate. AMP was rapidly incorporated into untreated yeast tRNA, while CMP was preferentially incorporated into phosphodiesterase-treated tRNA. The enzyme incorporated CMP at the same rate into phosphodiesterase-treated tRNA from yeast, *Neurospora*, or *E. coli*. Neither GTP nor UTP was used as substrates. The K_m values for ATP and CTP were 0.33 and 0.06 mM, respectively. The K_m for yeast tRNA (AMP incorporation) was 12 μM. Since the untreated yeast tRNA contained 12.4 nmol of active AMP-accepting tRNApCpC per mg of (M. Nazario, in preparation), the K_m for tRNApCpC was recalculated (assuming no inhibition by the inactive tRNAs) to be 4 μM. The K_m for phosphodiesterase-treated yeast tRNA (CMP incorporation) was 6.5 μM. The phosphodiesterase-treated tRNA contained 7.6 nmol of tRNApC and 3.5 nmol of tRNApN per mg. The procedures used to analyze the tRNAs, along with studies on the extent of anomalous incorporation of nucleotides, will be presented elsewhere.

Inhibition by One Nucleotide of the Incorporation of the Other. CTP inhibited the incorporation of AMP and ATP inhibited CMP incorporation. Competitive inhibition was observed in both cases. K_i values, calculated from Dixon plots (Dixon, 1953),were 0.33 mM for ATP and 0.07 mM for CTP. The results for ATP are illustrated in Figure 2, and for CTP in Figure 3.

The close agreement between the K_i and K_m values (see above) immediately suggested that the catalytic and inhibitory binding site(s) are the same. ATP and CTP could bind to a single site (with different affinities) or to two substrate-specific sites. In order to distinguish between these two possibilities, the inhibition of the enzyme by several nucleotide substrate analogs was studied. dATP, dCTP, and α,β-methyleneadenosine triphosphate were competitive inhibitors of AMP and CMP incorporation. Their K_i's, along with Lineweaver–Burk determinations of K_i for ATP and CTP, are presented in Table III. The single binding site alternative (see above) would be favored if very similar K_i's for a given nucleotide analog were obtained in the presence of either ATP or CTP. Since the K_i's for the nucleotide substrate analogs in the two assays were different (Table III), the results did not support the single nucleotide binding site model.

Discussion

The specific activity of tRNA nucleotidyl transferase found in extracts of *Neurospora* is from a few to several hundred times higher than reported for extracts of *E. coli* (Preiss *et al.*, 1961; Miller and Philipps, 1970; Carre *et al.*, 1970; Best and

TABLE II: Nucleic Acid and Nucleotide Specificity.[a]

Nucleic Acid (mg)	nmol Incorporated in 5 min			
	ATP	CTP	UTP	GTP
Yeast tRNA (0.36)	0.78	0.06	<0.01	<0.01
Phosphodiesterase-treated yeast tRNA (0.40)	0.01	0.31	<0.01	<0.01
rRNA (0.40)	<0.01	<0.01	<0.01	<0.01
"Sheared" calf thymus DNA (0.32)	<0.01	<0.01	<0.01	<0.01

[a] The assay conditions were those described under Materials and Methods. The UTP and GTP used had specific activities of 10 Ci/mol; their final concentration was 0.8 mM. The calf thymus DNA was sheared by forcing a solution (3.4 mg/ml) 50 times through a 16-gauge needle.

TABLE III: Inhibition Constants of Substrate Nucleotide Analogs.[a]

Inhibitor	AMP Incorp K_i (mM)	CMP Incorp K_i (mM)
dATP	0.9	1.9
dCTP	3.2	0.7
α,β-Methyleneadenosine triphosphate	2.4	6.5
CTP	0.06	
ATP		0.38

[a] K_i's were calculated from double-reciprocal plots (Lineweaver and Burk, 1934).

Novelli, 1971), yeast (Lebowitz *et al.*, 1966; Morris and Herbert, 1970), rat liver (Daniel and Littauer, 1963), rabbit liver (Deutscher, 1970), and muscle (Starr and Goldthwait, 1963). Very recently, purified tRNA nucleotidyltransferases with higher specific activities were reported (Sternbach *et al.*, 1971; Deutscher, 1972a). Although the *Neurospora* preparation is not homogeneous, several aminoacyl-tRNA ligases and a nuclease activity present in the DEAE-cellulose pool (fraction III) were eliminated by chromatography on hydroxylapatite (unpublished studies). The kinetic properties of the enzyme vary widely depending on the source. K_m values for ATP one order of magnitude higher (Starr and Goldthwait, 1963; Deutscher, 1972b) or lower (Furth *et al.*, 1961) have been reported. Important differences in the effects of one nucleotide on the incorporation of the other have also been recorded. Based mainly on the additive rates of incorporation of AMP and CMP, and the difference between the K_m and K_i values for CTP, Anthony *et al.* (1963) postulated separate active sites for ATP and CTP for the rabbit muscle enzyme; two CTP binding sites were suggested by a biphasic double-reciprocal plot; CTP competitively inhibited AMP incorporation whereas ATP stimulated CMP incorporation. Very similar results were obtained with a purified rabbit liver enzyme (Deutscher, 1972b).

No nucleotide interactions were observed with the *E. coli* W enzyme (Furth *et al.*, 1961) while Carre *et al.* (1970), using enzyme from *E. coli* MRE 600, reported that ATP was a noncompetitive inhibitor of CMP incorporation, and CTP was a noncompetitive inhibitor of AMP incorporation; they concluded that the active sites for ATP and CTP were different. Best and Novelli (1971) observed competitive inhibition of AMP incorporation by CTP; the K_m for ATP was 0.15 mM and the K_i for CTP was 0.18 mM; the K_m for CTP was 9 μM. Miller and Philipps (1971) observed competitive inhibition by ATP and by CTP; again, the K_m values were 33 and 160 times lower than the corresponding K_i's.

In the case of the *Neurospora* enzyme, the K_i and K_m values for a given nucleotide substrate were essentially the same. Assuming that K_m values represent binding constants, the results indicate that similar events take place when a given nucleotide (say ATP) binds as substrate or as inhibitor, suggesting the identity of the catalytic and inhibitory sites. The possibility that ATP and CTP are bound to a single site was explored by determinations of K_i for three nucleotide substrate analogs that were found to be competitive inhibitors. The results in Table III show that the adenine analogs (dATP and α,β-methyleneadenosine triphosphate) have a higher affinity for the enzyme when they compete with ATP. Likewise, the affinity of dCTP is higher in the presence of CTP. The finding of base specificity in the inhibitory effects of the nucleotide analogs does not support (although it does not rule out) the single nucleotide binding site alternative. If multiple sites are considered (Deutscher, 1972b), the inhibitory effect of CTP could be described in terms of steric hindrance of the tRNA substrate by the inhibitor. An explanation of the inhibition by ATP, on the other hand, may require the postulation of an enzyme–inhibitor complex with a reduced affinity for the nucleotide substrate at the CTP site. Additional physical and kinetic studies will be necessary before a detailed model can be proposed.

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