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Purification of Cysteine Transfer Ribonucleic Acid of Bakers' Yeast†

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ABSTRACT: A procedure for the purification of cysteine transfer ribonucleic acid ($tRNA^{Cys}$) of Bakers' yeast has been developed which is based upon the reaction of the sulfhydryl moiety of cysteinyl-$tRNA^{Cys}$ with an organomercurial–polysaccharide. tRNA charged with L-[^{35}S]cysteine was prepared by the reaction of the amino acid with heterogeneous tRNA of yeast in the presence of yeast L-cysteinyl-tRNA synthetase. Preliminary purification of the RNA was necessary to remove protein and unreacted [^{35}S]cysteine from the mixture prior to reaction of the cysteinyl-$tRNA^{Cys}$ with the organomercurial–polysaccharide column material. The $tRNA^{Cys}$ was recovered from the column by elution with 0.02 M Tris-HCl (pH 7.2). A 43-fold purification of the $tRNA^{Cys}$ was accomplished by this procedure. Attempts to charge the purified RNA with other amino acids gave negative results.

The complex function of a specific tRNA would be difficult to study without first having at hand the means of obtaining the tRNA in relatively pure form. Purification of a particular tRNA is complicated by the fact that all species of tRNA are relatively similar in nature and have molecular weights of approximately 25,000 (Litt and Ingram, 1964). This report describes a method for the purification from yeast of a tRNA specific for the amino acid cysteine. The method is unique in that it relies upon the chemical nature of the amino acid rather than upon the nature of the specific tRNA. After reaction of cysteine with unfractionated tRNA in the presence of the L-cysteinyl-tRNA synthetase, the cysteinyl-tRNA is selectively bound to an organomercurial–polysaccharide material through the sulfhydryl group of the tRNA-bound amino acid. tRNAs specific for other amino acids are removed by decantation and $tRNA^{Cys}$ of high purity is eluted. A preliminary report of certain areas of this work has been presented (James *et al.*, 1967).

Materials and Methods

Preparation of Enzyme Fraction from Baker's Yeast. A crude soluble fraction of Bakers' yeast was prepared by a modification of the method of James and Bucovaz (1966). Approximately 1400 g of Bakers' yeast (Federal Yeast Corp.) was frozen in an ether–CO_2 mixture for 8 hr to break the cells. After removing the ether by aspiration, the resulting homogenate was thawed and extracted with 15 g of KCl. Following the KCl extraction, the homogenate was centrifuged at 105,000 · *g* for 1 hr in a Spinco Model L centrifuge with an analytical rotor No. 40. The supernatant liquid obtained was the crude enzyme fraction.

Purification of the Crude Enzyme Fraction. Approximately 350 ml of the crude enzyme fraction was adjusted to 10% saturation with $(NH_4)_2SO_4$ (65 mg of $(NH_4)_2SO_4$/ml of solution) and the resulting precipitate, which contained no major enzyme activity, was discarded. The supernatant liquid was increased to 75% saturation with $(NH_4)_2SO_4$. After centrifugation the supernatant liquid was discarded and the precipitate was dissolved in 100 ml of 0.01 M Tris-HCl (pH 7.5). The redissolved precipitate was subdivided into three fractions (0–35% precipitate, 35–60% precipitate, and 60% supernatant fraction) by additions of $(NH_4)_2SO_4$. The resulting precipitates were dissolved in 0.01 M Tris-HCl (pH 7.5) and all three fractions were dialyzed against 0.01 M Tris-HCl (pH 7.5) for 15 hr to remove $(NH_4)_2SO_4$ and other small molecular

† From the Department of Biochemistry, University of Tennessee Medical Units, Memphis, Tennessee 38103. *Received June 5, 1972.* This investigation was supported in part by Public Health Service Research Grant AM-09131 from the National Institute of Arthritis and Metabolic Diseases.

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weight components. The 35–60% precipitate, which contained the cysteinyl-tRNA synthetase and enzyme activity for 19 other naturally occurring amino acids, was used in this study. The temperature was maintained at 0–5° throughout the purification procedure.

tRNA. tRNA of Bakers' yeast, obtained commercially (General Biochemicals), was used as the starting material for purification of tRNA^{Cys}.

Reaction of L-[^{35}S]*Cysteine with tRNA.* The reaction mixture contained the following components: 3.0 μmoles of tRNA, 0.4 ml (3.26 mg of protein) of the enzyme fraction, 0.396 μmole of L-[^{35}S]cysteine (616,883 cpm/μmole), 3.0 mM ATP, 6.0 mM $MgCl_2$, and 100 mM Tris-HCl (pH 7.5). The mixtures were incubated at 37° for 30 min and the tRNA was isolated for analysis as indicated below.

Recovery and Analysis of the tRNA. A modification of the method of Gierer and Schramm (1956) and Kirby (1956) was used to recover the tRNA from the incubation mixture. After recovery the tRNA was reprecipitated with 0.1 volume of 20% potassium acetate solution and 3.0 volumes of ethanol, the tRNA was dissolved in 3 ml of water and centrifuged, and the supernatant liquid was dialyzed for 15 hr against three changes of distilled water at 5°. The dialyzed material was assayed for RNA content and radioactivity.

Stripping of tRNA. tRNA was stripped of bound amino acids by incubation for 40 min at 37° in 0.5 M Tris-HCl (pH 9) (Nathans and Lipmann, 1961). The "stripped" RNA was dialyzed for 15 hr in 0.01 M Tris-HCl (pH 7.5).

Preparation of Organomercurial–Polysaccharide. The organomercurial–polysaccharide column material was prepared according to the method of Eldjarn and Jellum (1963) for the separation of proteins containing SH groups using Sephadex G-25 as the supporting medium, and 3,6-bis(acetatomercurimethyl)dioxane as the active material.

Analytical Methods. Total protein was determined by the method of Lowry *et al.* (1951). The orcinol method of Hendler (1956), a colorimetric determination using the Klett spectrophotometer (filter 52), and ultraviolet analysis at 260 mμ were used to measure the content of tRNA. The 3,6-bis(acetatomercurialmethyl)dioxane was assayed by adding 4.5 ml of 0.1 M KI to 0.5 ml of the solution tested. Radioactivity was measured in a Packard Tri-Carb scintillation counter with correction for quenching and background.

Results and Discussion

To prepare an adequate amount of [^{35}S]Cys-tRNA^{Cys} for isolation, tRNA was charged with L-[^{35}S]cysteine as described under Materials and Methods. After incubation for 30 min at 37°, the tRNA was recovered and dialyzed for 15 hr against three changes of water. The labeled tRNA was pooled, passed through a column of Sephadex G-25 to remove trace amounts of free [^{35}S]cysteine, and assayed for content of bound L-[^{35}S]cysteine. A total of 1.23 μmoles of tRNA with a specific activity of 14,250 cpm/μmole (representing 0.0231 μmole of bound cysteine) was recovered from the column. Undoubtedly, the quantity of tRNA^{Cys} determined is a conservative figure, due to probable hydrolysis of the ester linkage binding cysteine with tRNA (see Table I).

The conditions for binding cysteinyl-tRNA^{Cys} with organomercurial–polysaccharide were established by batchwise experiments carried out in centrifuge tubes for 5, 10, and 15 hr at 5°. Figure 1 shows that the attachment of cysteinyl-tRNA^{Cys} to the organomercurial–polysaccharide is more efficient in water (pH 5.5) than in either 0.01 M phosphate buffer (pH 7.5) or 0.01 M bicarbonate (pH 8). The cysteinyl-tRNA^{Cys}–organomercurial–polysaccharide complex was washed repeatedly with water to remove tRNAs specific for other amino acids, and the cysteinyl-tRNA^{Cys} was eluted from the organomercurial–polysaccharide material with 0.02 M Tris (pH 7.2).

TABLE I: Effect of Lyophilization and Dialysis on Fractions from Column.

Fraction No.[a]	Assay of Components	Lyophilization and Dialysis: Before	Lyophilization and Dialysis: After
	Total RNA (μmole)	0.0217	0.0217
103–138	Total radioactivity (cpm)	12,590	12,230
	KI assay[b]	+	0
	Total RNA (μmole)	0	0
140–170	Total radioactivity (cpm)	780	0
	KI assay	+	0
	Total RNA (μmole)	0.0004	0.0004
180–220	Total radioactivity (cpm)	0	0
	KI assay	+	0

[a] See Figure 2. [b] The KI assay was used to determine the 3,6-bis(acetatomercurimethyl)dioxane. A plus sign indicates the presence and a zero sign the absence of the material. The procedure used for the assay is described in the text.

The [^{35}S]cysteinyl-tRNA was mixed in distilled water with a sufficient amount of the organomercurial–polysaccharide column material to pack a column of 10 × 40 cm. After mixing for 10–15 hr at 5°, the organomercurial–polysaccharide material was washed several times with distilled water by sedimentation and decantation. Figure 2 shows the pattern of elution of cysteinyl-tRNA from the organomercurial–polysaccharide column. The chromatography was carried

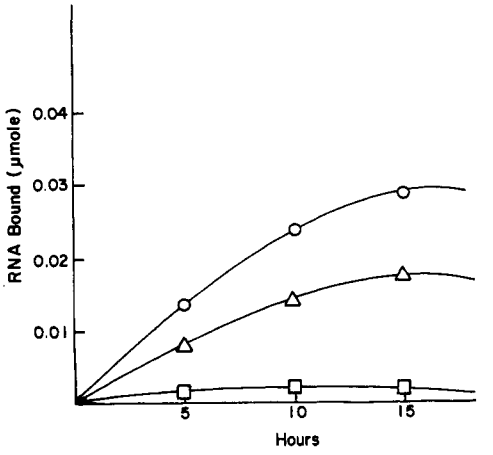

FIGURE 1: Binding of [^{35}S]cysteinyl-tRNA^{Cys} with the organomercurial–polysaccharide column material in water (pH 5.5) (○), 0.01 M sodium phosphate buffer (pH 7.5) (△), and 0.001 M sodium bicarbonate (pH 8.0) (□). The recovery of [^{35}S]cysteinyl-tRNA^{Cys} was determined using 10-ml volumes of column material. The volumes expressed are for a column 5 × 40 cm.

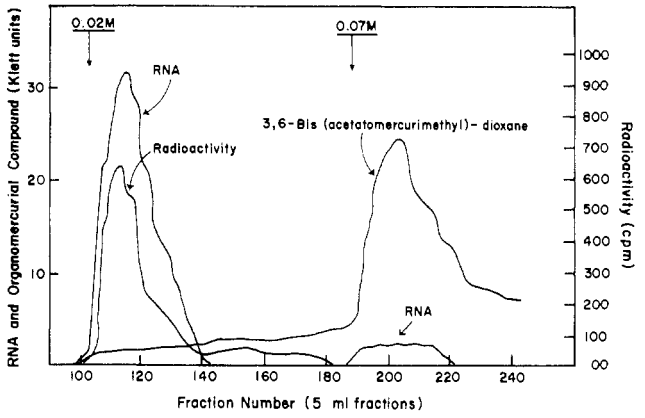

FIGURE 2: Elution pattern of [^{35}S]cysteinyl-tRNA on an organomercurial–polysaccharide column.

out at room temperature. The flow rate was maintained at 1 ml/min. Initially, 520 ml of distilled water was passed through the column. Only small quantities of RNA, radioactivity, or organomercurial compound, representing unbound components not removed by previous washings of the column material (*e.g.*, 3,6-bis(acetatomercurimethyl)-dioxane), were detected in the water eluate (fractions 1–102). Following the elution with water, 400 ml of 0.02 M Tris-HCl (pH 7.2) was added to the column (fractions 103–189). The [^{35}S]cysteinyl-tRNA^{Cys} was recovered in fractions 103–140, and most of the radioactivity occurred coincidentally with the RNA. Small amounts of radioactivity, however, were found in fractions 141–179, and these small quantities probably represented [^{35}S]cysteine released from the RNA by hydrolysis. Finally, the concentration of Tris-HCl was increased to 0.07 M, removing the 3,6-bis(acetatomercurimethyl)-dioxane from the column (fractions 188–230). No radioactivity was associated with the small amount of RNA that was present in these fractions. From the data of Figure 2 it can be inferred that the Hg–S bondings of the cysteinyl-tRNA (fractions 106–140) are more easily split than the binding between the SH-Sephadex and the organomercurial compound (fractions 188–230, Figure 2). Because of its affinity for mercury, Tris at low concentrations (0.02 M) ruptures the Hg–S–cysteinyl-tRNA linkage, and at higher concentrations (0.07 M) cleaves the organomercurial–SH-Sephadex linkage.

Fractions (103–138, 140–170, and 180–220) collected from the organomercurial–polysaccharide column (Figure 2) were pooled, lyophilized, and then dialyzed for 15 hr at 5° against distilled water (pH 5.5). The pooled fractions were assayed before and after dialysis and lyophilization (Table I). Fractions 103–138 and 180–220 contained 0.0217 and 0.0004 μmole of RNA, respectively. The RNA content remained unchanged in these pooled fractions after dialysis. No RNA was detected in fractions 140–170. The majority of the radioactivity (*i.e.*, [^{35}S]cysteine bound to RNA) was present in fractions 103–138; a lesser amount was present in 140–170, and none in fractions 180–220. After lyophilization and dialysis, some of the radioactivity was lost from fractions 103 to 138, and all detectable radioactivity was lost from fractions 140 to 170. The loss of radioactivity from fractions 103 to 138 was probably due to hydrolysis during lyophilization and dialysis. A small amount of the 3,6-bis(acetatomercurimethyl)dioxane was detected in fractions eluted with 0.02 M Tris-HCl (fractions 103–138 and 140–170), indicating that some hydrolysis of the organomercurial compound from SH-Sephadex takes place in 0.02 M Tris-HCl. Fractions 180–220 were eluted with 0.07 M Tris, which ruptured the bond between the organomercurial compound and SH-Sephadex. Usually, after extensive dialysis, all detectable organomercurial compound was removed (Table I), but occasionally traces of the compound remained in fractions 180–220, which may be due to the large amount of 3,6-bis(acetatomercurimethyl)dioxane in these fractions and the physical limitation of the procedure of dialysis used.

TABLE II: Effect of Column Size and Quantity of RNA on Recovery.

Expt No.	Column Size (cm)	[^{35}S]Cysteinyl-tRNA Added (μmole)	[^{35}S]Cysteinyl-tRNA Recovd (μmole)
1	10 × 40	0.030	0.022
2	5 × 40	0.030	0.023
3	10 × 40	0.015	0.010

TABLE III: Assay for Purity of tRNA^{Cys}.[a]

Expt No.	Reaction Mixture Components	Radioactivity RNA Added (μmole)	Radioactivity Sp Act.[b] (cpm/μmole of tRNA)
1	[^{35}S]Cysteine + RNA (commercial) + ATP	0.480	14,139
2	[^{35}S]Cysteine + RNA (purified) + ATP	0.0052	611,666
3	[^{35}S]Cysteine + RNA (purified) + ATP	0.010	610,092

[a] Each reaction mixture contained 0.396 μmole of [^{35}S]-cysteine (616,883 cpm/μmole); the quantity of tRNA either commercial or purified as indicated in the table; and the other components given under Materials and Methods for the binding of [^{35}S]cysteine to RNA. After incubation for 30 min at 37° the reactions were stopped by the method described under Materials and Methods. To each tube containing purified tRNA 0.400 μmole of commercial RNA was added as a carrier to facilitate recovery of the ^{35}S-labeled purified tRNA. [b] Specific activity represents net cpm/μmole.

Support for specific binding of the SH group of cysteinyl-tRNA^{Cys} to the organomercurial–polysaccharide column material was provided by studies in which the amounts of column material or tRNA were varied and the RNA recovered in each experiment was measured. As seen for expt 1 and 2 of Table II, the amount of tRNA recovered is not dependent on the quantity of column material within the range shown. If binding were not of a specific nature, *i.e.*, not with the SH group of cysteinyl-tRNA^{Cys}, the amonnt of tRNA recovered should vary directly with the size of the column. Thus, the cysteinyl-tRNA^{Cys} recovered must depend on the amount added to the column. Experiments 1 and 3 of Table II show that the amount of tRNA recovered is directly related to the amount added to the column material.

The data of Table III compare the extent of labeling of

TABLE IV: Comparison of Data on Extent of Purification of RNA^{Cys}.

tRNA	μmoles of Cys/ μmole of RNA
RNA (commercial) (Table I)	0.0231
RNA (purified) (Table III)	0.9903
RNA (commercial) (Table III)	0.0229

tRNA recovered from the organomercurial–polysaccharide column with that for commericial tRNA. Experiment 1 contained commercial tRNA. Experiments 2 and 3 contained purified tRNA. A comparison of the net specific activities obtained in expt 2 and 3 shows that agreement was obtained for [^{35}S]cysteine transferred to RNA when either 0.0052 or 0.01 μmole of the purified $tRNA^{Cys}$ was used. From a comparison of the net specific activities of the purified RNA (expt 2 and 3) with the net specific activity of commercial RNA (expt 1), it was estimated that the $tRNA^{Cys}$ had been purified approximately 43-fold. In other studies not shown, a mixture of [^{14}C]amino acids (arginine, histidine, isoleucine, methionine, proline, threonine, and valine) could not be transferred to the purified RNA.

Calculations were made on the assumption that tRNA has a molecular weight of 25,000. Because 1.25 μmoles of tRNA per milliliter produces 1 unit of absorbancy at 260 mμ (Berg *et al.*, 1961), the amino acid acceptor activity and the absorbancy were used to estimate purity. Table IV shows the accumulated results of this purification procedure. The commercial RNA, from which the purified $tRNA^{Cys}$ was obtained, compared favorably with regard to the amount of cysteine transferred per μmole of RNA in expt 1 of Table III. Approximately 0.023 μmole of [^{35}S]cysteine/mole of RNA was transferred in each case. From these data, it was calculated that 2.3% of the commercial RNA was charged with cysteine. The recovered RNA could be charged with 0.9903 μmole of cysteine/μmole of RNA (Table III), indicating approximately a 43-fold purification of $tRNA^{Cys}$.

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Mixed Conformations of Deoxyribonucleic Acid in Intact Chromatin Isolated by Various Preparative Methods†

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ABSTRACT: The optical and analytical characteristics of calf thymus nucleohistone (TNH) isolated by a variety of methods have been examined. In general, the values of ϵ_P (the extinction coefficient at the maximum) and the protein/DNA ratios of the products obtained from these various methods are comparable. The circular dichroism (CD) properties and the melting behavior, however, differ in a significant fashion. The circular dichroism data taken together with the thermal melting profiles are best interpreted in terms of the presence of two discrete conformations of DNA in TNH. Part of the DNA duplex is exposed or accessible to the solvent environment—either completely or partially—and is in the B conformation. The remainder of the base pairs and ribophosphate backbone is protected from the solvent environment by efficient histone coverage and is in the C conformation. Correlation with other data in the literature suggests that these latter sections, as a consequence of the C conformation and effective charge neutralization, may also be in a super-coiled form. Addition of divalent ions increases the per cent C character at the expense of the B, whereas the addition of NaEDTA has the reverse effect. These ions may be an integral part of the native structure of TNH *in vivo*. Variations in the CD and melting properties of the various preparations can be attributed to variations in the removal of ions from the TNH in the early stages of isolation from the tissue.

The structure of the chromatin of eucaryotes has recently become the subject of intense investigational activity. (For comprehensive reviews, see Bonner *et al.* (1968b); Stellwagen and Cole (1969), Hearst and Botchan (1970), and DeLange and Smith (1971).) These investigations have revealed that it is a multicomponent entity consisting of at least two classes

† From the Department of Biological Chemistry, College of Medicine, University of Illinois, Chicago, Illinois 60612. *Received June 8, 1972.* Taken in part from the Ph.D. thesis of Roger S. Johnson. This investigation was supported by Grant GB 24550 from the National Science Foundation.