CHEMICAL MODIFICATIONS OF TRANSFER RNA SPECIES. HEAVY ATOM DERIVATIZATION OF AMINOACYL tRNA.

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Received June 12, 1972

SUMMARY

Specific heavy atom derivatization of valyl and arginyl tRNA's from E. coli has been effected by the use of the N-hydroxysuccinimide esters of certain carboxylic acids. The derivatized tRNA's have been separated from underivatized material and shown to be stable under the conditions required for crystallization.

INTRODUCTION

Several approaches have been employed for the introduction of heavy atoms into transfer RNA to create species which may serve as potential crystalline isomorphous derivatives. The addition of metal ions has been effected to binding sites either preexisiting in the tRNA molecule (1-3) or chemically and enzymatically introduced (4-6). In only one case has a single heavy atom been covalently introduced into otherwise native tRNA (7,8).

The design of heavy atom derivatives substituted at a single, predetermined site requires the presence of a unique functionality in the transfer RNA. One such functionality is constituted by the amino acid moiety on aminoacyl tRNA. Lapidot and his coworkers demonstrated that this group could be specifically acylated with additional amino acids or alkyl carboxylic acids by the use of the intermediate N-hydroxysuccinimide esters, as shown in Scheme 1 (9-11). The peptidyl and acylated aminoacyl tRNA's formed by this procedure were noted to be rather more stable to hydrolysis than were the underivatized aminoacyl tRNA's.

We report a procedure, based on the work of Lapidot, for the derivatization of tRNA's with a variety of heavy atoms. The procedure involved treatment of aminoacyl tRNA with the N-hydroxysuccinimide ester of a carboxylic acid derivative containing a heavy atom, followed by

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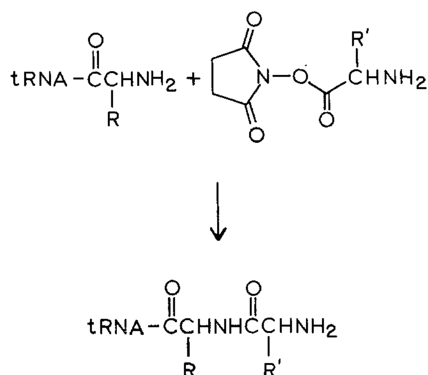
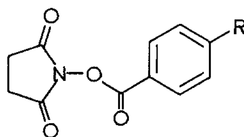


Scheme 1. Derivatization of aminoacyl tRNA to form peptidyl tRNA.



1, R=H
2, R=HgCl
3, R=I

purification of the derivatized tRNA on benzoylated diethylaminoethyl-cellulose (BD cellulose). Three heavy atom derivatives were utilized. These were the p-chloromercuribenzoic acid (2), p-iodobenzoic acid (3), and iodoacetic acid (4) esters of N-hydroxysuccinimide. The derivatized tRNA represents the first reported heavy atom derivatization of an aminoacyl tRNA and is of additional interest from a crystallographic standpoint in the sense that aminoacyl tRNA may have a different tertiary structure than tRNA in the absence of the amino acid (2).

MATERIALS AND METHODS

p-Chloromercuribenzoic acid (PCMB) was purchased from K&K Laboratories. N-hydroxysuccinimide and N,N'-dicyclohexylcarbodiimide were purchased from Aldrich Chemical Company. The latter was purified by distillation at diminished pressure prior to use. [^{14}C]-arginine and valine were purchased from Schwarz-Mann. BD cellulose was synthesized by Dr. M. Miyazaki from Whatman DEAE cellulose (DE 23) by the procedure of Gillam, et al. (12). The synthesis of the N-hydroxysuccinimide esters of carboxylic acids was effected by analogy with the procedure of

Lapidot, Rappoport and Wolman (10).

Synthesis of the N-hydroxysuccinimide ester of benzoic acid. To 10 ml of dry ethyl acetate, containing 3.66 g (30 mmoles) of benzoic acid, was added 3.45 g (30 mmoles) of N-hydroxysuccinimide. To this solution was added 6.18 g (30 mmoles) of N,N'-dicyclohexylcarbodiimide dissolved in 10 ml of dry ethyl acetate. The combined solution was stirred for 0.5 hr at room temperature and the resulting suspension was filtered. Concentration of the ethyl acetate afforded a solid residue which was recrystallized from ethanol to afford white crystals, yield 4.0 g (61%), mp 129.5 - 131.5°; nmr δ values from tetramethylsilane (dimethylsulfoxide-d_6): 3.00 (4H, s), 8.05 (5H, m).

Anal. Calcd. for $C_{11}H_9NO_4$: C, 60.28; H, 4.14. Found: C, 60.01; H, 4.17.

The syntheses of the esters of p-iodobenzoic acid and p-chloromercuribenzoic acid were effected by similar procedures.

Synthesis of the N-hydroxysuccinimide ester of iodoacetic acid. To 10 ml of dry dioxane containing 5.58 g (30 mmoles) of iodoacetic acid was added 3.45 g (30 mmoles) of N-hydroxysuccinimide. To this solution was added 6.18 g (30 mmoles) of N,N'-dicyclohexylcarbodiimide dissolved in 10 ml of dry dioxane. The combined solution was stirred for 0.5 hr at room temperature and filtered to remove dicyclohexylurea. The filtrate was concentrated to a small volume under diminished pressure and the resulting suspension was cooled and filtered. Recrystallization of the solid from ethanol afforded white crystals of the ester, yield 5.8 g (77%), mp 145-146°.

Anal. Calcd. for $C_6H_6NO_4I$: C, 25.46; H, 2.14. Found: C, 25.76; H, 2.31.

Aminoacylation of tRNA. The reaction mixture contained 0.001 N ATP, 0.004 N CTP, 0.1 N PIPES (piperazine-N,N'-bis-(2-ethanesulfonic acid)) buffer (NH_4^+ form, pH 7.0), 5×10^{-4} N EDTA, 0.01 N KCl, 0.01 N $MgCl_2$, 5×10^{-5} N [^{14}C]-valine, specific activity 50 μCi/μmole. Crude tRNA was added to a concentration of 0.8 A_{260} units per ml, and the reaction was initiated by the addition of homologous, partially fractionated enzyme in 50% glycerol (approximately 15% of the total volume). After the incorporation was complete, the reaction was stopped by the addition of 2×10^{-3} meq of cetyltrimethylammonium bromide (CTAB) per A_{260} unit of tRNA or by the addition of 1.5 volumes of 88% phenol. After ethanol precipitation, the tRNA was dried and redissolved in 0.01 N $MgCl_2$, 0.01 N acetate- sodium acetate buffer, pH 4.5.

Acylation of aminoacyl tRNA. Initial attempts (13), which utilized a

modification of the procedure of Gillam, et al. (14), did not result in formation of the desired derivative, presumably due to differences in the solubility of the derivatizing agents. Therefore, either the two phase system of Lapidot, et al. (9), modified to ensure solubility of the N-hydroxysuccinimide esters, or a homogeneous system utilizing lesser amounts of N, N-dimethylformamide (DMF) was employed. The general procedures were as follows.

Method A. The aminoacyl tRNA (15 A_{260} units, dissolved in 0.1 ml acetate buffer, 0.1 N, pH 4.5) was added to 0.9 ml of DMF containing 1-20 mg of N-hydroxysuccinimide ester. The tubes were shaken for 24 hr at room temperature. The precipitate was then isolated by centrifugation and washed with 6 ml of DMF. tRNA was dissolved in 0.5 ml of water and precipitated with two volumes of cold 95% ethanol. The precipitates were dried under vacuum.

Method B. The aminoacyl tRNA was dissolved in 0.1 N triethanolamine hydrochloride, pH 4.5. To the solution was added 0.67 volumes of DMF containing the appropriate N-hydroxysuccinimide ester (100 μmoles/ml). The pH was adjusted to 8 by the addition of 0.5 N KOH solution, and the reaction mixture was shaken gently at room temperature for 4 hr. tRNA was isolated by precipitation with ethanol.

The extent of reaction of the aminoacyl tRNA was determined by alkaline hydrolysis of the aminoacyl ester bond (0.5 N KOH solution, 30 min.), followed by chromatography of the hydrolysate on Whatman DE 81 anion exchange paper, elution with water.

RESULTS AND DISCUSSION

The results of the derivatization of the [^{14}C]-valyl tRNA contained in unfractionated E. coli tRNA are shown in Table 1. Similar results were obtained with [^{14}C]-arginyl tRNA. All of the valine derivatives had R_f=0 on DEAE paper, elution with water, either because of low solubility of the complex or the negative charge on the amino acid derivative. p-Iodobenzoylvalyl tRNA may be quantitatively separated from the bulk of the tRNA and from the underivatized [^{14}C]-valyl tRNA by chromatography on BD cellulose, elution with salt and ethanol gradients, as described by Gillam, et al. (14). The material eluted with ethanol gave no free amino acid upon hydrolysis, while samples of material from the salt-eluted peaks gave no counts at R_f=0 when further analyzed by chromatography on DE 81 paper, elution with water (Table 1).

The stability of the derivatized aminoacyl tRNA under crystallization conditions was investigated by dissolving the derivatized tRNA in a standard

TABLE 1. Chromatography of the Alkaline Hydrolysis Product of Derivatized Valyl tRNA[a]

Compound	Counts[b] (cpm) in CTAB-precipitable material at: $R_f = 0$[c]	$R_f = 0.6$[d]
1	17,700	10,300
2	5,200	13,800
3	15,400	27,000
4	37,000	2,300
control (no derivatizing agent)	4,100	30,500
3, after purification on BD cellulose	1,200	40

[a]Derivatization effected by Method B (see Materials and Methods). [b]Counted in toluene-based scintillator on paper cut from DE 81 chromatograms. [c]Counts corresponding to derivatized aminoacyl tRNA. [d]Counts corresponding to underivatized aminoacyl tRNA. [e]Method A (see Materials and Methods) afforded up to 45% derivatization.

TABLE 2. Stability of Derivatized tRNA.

Aminoacyl tRNA's	CTAB-precipitable counts (cpm)[a] frozen	8°[b]	25°[b]
Benzoyl[^{14}C]arg tRNA	1729	763(44%)	449(26%)
PCMB[^{14}C]arg tRNA	1730	1131(65%)	819(47%)
Benzoyl[^{14}C]val tRNA	2223	1712(77%)	1497(68%)
PCMB[^{14}C]val tRNA	2288	2310(100%)	2215(96%)

[a] After twelve days. [b] Maintained under crystallizing conditions, as defined in ref. 15.

medium under conditions employed for crystallization in our laboratory (15). After 12 days, the remaining aminoacyl tRNA was precipitated with CTAB

and counted on paper discs. The results, corresponding to derivatizing agents 1 and 2 for valyl and arginyl tRNA's, are shown in Table 2. They indicated the greater stability of the PCMB derivative over the benzoyl derivative and the general stability of the derivatized tRNA's under crystallization conditions, which is sufficient to permit their utilization during the 3-14 day time period required for crystal growth.

ACKNOWLEDGMENTS

We thank Drs. A. H. Mehler and K. Chakraburrty for help with the initial experiments. We thank Dr. M. Miyazaki for the preparation of BD-cellulose and M. A. Helmes for technical assistance. Supported by NIH research grant GM-12395 and NSF research grant GB-31212. FJS is an NIH trainee.

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