THE ENZYMATIC CONVERSION OF GLUTAMINYL-tRNA TO PYRROLIDONE CARBOXYLATE-tRNA

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SUMMARY. The formation of pyrrolidone carboxylate-tRNA (PCA-tRNA) from E. coli glutaminyl-tRNA is readily catalyzed by glutamine cyclotransferase, an enzyme of papaya latex. The glutamine is cyclized without apparent cleavage of the aminoacyl ester and activity toward free glutamine is slight. Glutamic acid and glutamyl-tRNA are not substrates. The enzyme is reported to have activity toward N-glutaminyl residues in synthetic peptides and thus is active toward glutamine in ester linkage to tRNA as well as in peptide linkage. Since PCA is not directly incorporated into proteins, these findings suggest that naturally occurring PCA (which exists exclusively at the NH_2-terminal) may be derived from PCA-$tRNA^{Gln}$.

Several naturally occurring polypeptides and proteins contain pyrrolidone carboxylic acid (pyrrolid-2-one carboxylic acid, pyroglutamic acid, PCA) as their amino-terminal residue (1,2). Free PCA is not incorporated into protein (3,4), suggesting that polypeptide-bound PCA is derived from another amino acid, probably glutamic acid or glutamine. This conversion may occur on the aminoacyl- (or peptidyl-) transfer RNA or on the polypeptide. Messer and Otteson (5,6) have described an enzyme (glutamine cyclotransferase, GCT) from papaya latex which catalyzes the conversion of synthetic N-glutaminyl peptides to the corresponding PCA-peptides. To test the hypothesis that PCA formation may occur on aminoacyl-tRNA, we tested GCT for activity on glutaminyl-tRNA and found that it catalyzes the quantitative conversion of glutaminyl-tRNA to PCA-tRNA without cleavage of the aminoacyl ester.

EXPERIMENTAL. Materials - Frozen E. coli W was the source of mixed tRNA (7) and aminoacyl-tRNA synthetase (8). The synthetase preparation was stored at -20° in 250 mM K phosphate (pH 6.5), 1 mM $MgCl_2$, 6 mM mercaptoethanol made 25% in glycerol. Papaya latex glutamine cyclotransferase (fraction 3, 105-fold purified) was a gift of Dr. M. Messer (6). ^{14}C-L-PCA was a gift of Dr. R. Doolittle. ^{14}C-L-glutamine (41.3 μc/μmole) and ^{14}C-L-glutamic acid (130 μc/μmole) were obtained from Nuclear Chicago. L-glutamine and L-glutamic acid were from Mann Research and L-PCA from K and K Laboratories.

Acylation of tRNA was performed in a reaction mixture containing 100 mM K cacodylate (pH 6.9), 10 mM Mg acetate, 5 mM K ATP, 0.5 mM dithiothreitol, 0.05 mM 19 ^{12}C-amino acids other than glutamine, 0.05 mM ^{14}C-glutamine, 6 mg /ml tRNA and 1.4 mg /ml synthetase preparation. The reaction was incubated 20 minutes at 37°, chilled on ice and extracted three times with an equal volume of phenol saturated with 0.1 M Na acetate, pH 5.0. The ^{14}C-glutaminyl-tRNA was isolated from the aqueous phase by chromatography on Sephadex G-25 using 0.1 mM Na cacodylate pH 5.5 as eluent. Pooled fractions were lyophilized and stored at -20° under desiccation. The tRNA was 3.8% (mole/mole x 100) acylated with ^{14}C-amino acid and was uncontaminated with ATP or free amino acids.

Assay - The activity of GCT was assayed at 23° in 0.05 ml reaction mixtures containing 100 mM tris-HCl (pH 7.0), 200 µg/ml enzyme and various substrates. Zero time controls were frozen in dry ice immediately upon addition of substrate. After incubation, reaction mixtures were alkalinized (for deacylation) with KOH to pH 12-13 for 30 seconds at room temperature, and brought to pH 5-5.5 with acetic acid. Samples were applied to Whatman 3 MM paper for electrophoretic assay. For column assay, the samples were diluted to 0.55 ml with water and filtered through a column (0.5 x 2.5 cm) of Dowex 50 W (X-8, 100-200 mesh, H^+ form, Biorad) previously washed with water. The effluent from three 0.5 ml water washes was pooled and counted.

Analysis - Amino acids including PCA were separated (2) and detected (9) on paper electropherograms. Radioactivity bound to aminoacyl-tRNA was assayed by precipitation of the RNA with ice cold 5% TCA and collection of the precipitate on glass fiber filters. Radioactivity measurements were performed with a Beckman Liquid scintillation spectrometer. Paper strips cut at 1 cm intervals and dry glass fiber filters were counted directly in PPO-POPOP-toluene mixtures. Aqueous solutions were dissolved in 10 ml of a PPO-naphthalene-dioxane mixture for counting.

RESULTS. GCT action on free amino acids - Glutamine, glutamic acid and PCA were incubated with enzyme for 0 and 60 minutes, and the reaction mixtures, after alkali treatment, were subjected to paper electrophoresis. Distribution of radioactivity on the electropherograms is shown in Table I. The zero time values are nearly identical with those obtained from direct electrophoresis of the amino acids, indicating that the amino acids are stable to the experimental procedure. As reported by Messer and Otteson (5), GCT has some activity toward free glutamine, but is without effect on glutamate or PCA.

The results observed by paper electrophoresis were confirmed by use of the Dowex 50 column assay. Reaction mixtures with and without enzyme were incubated 30 minutes and assayed as described in the Experimental section. The data of Table II demonstrate the action of the enzyme on free glutamine, the lack of effect on free glutamic acid and PCA, and validate the use of the column method for the assay of PCA in the presence of glutamine and glutamic acid.

GCT action on glutaminyl-tRNA - Incubation mixtures containing glutaminyl-tRNA were incubated with and without enzyme for 30 minutes. An aliquot was removed for TCA precipitation and the remainder was deacylated and passed through Dowex 50 (Table III). Identical values for TCA-precipitable radioactivity were observed in reaction mixtures incubated with and without enzyme, demonstrating the absence of significant GCT-catalyzed deacylation during incubation. Less than 1% of the original TCA-precipitable radioactivity was detected after alkali treatment. An increase in radioactivity emerging from the column is seen in reaction mixtures incubated with enzyme. The radioactivity appearing in the column effluents of the enzyme-containing reactions was demonstrated to be exclusively in PCA by paper electrophoresis.

To demonstrate that the action of the enzyme was on glutaminyl-tRNA and not on free glutamine, the acylated tRNA was incubated with GCT and then isolated from the reaction mixtures by ethanol precipitation prior to column assay (Table IV). Significant radioactivity was observed only in those cases in which the aminoacyl-tRNA was isolated from GCT-containing mixtures, indicating that the product of the reaction is PCA-tRNA. Electrophoresis of the column effluents from enzyme-containing reactions again yielded PCA exclusively.

The crude E. coli enzyme preparation used to acylate the tRNA probably contains glutaminase (10) and, in addition, contains phosphate ions which are known to accelerate glutaminase activity (11). Therefore, despite the presence of ^{12}C-glutamate in the acylation reaction mixture, a significant proportion of the radioactivity bound to the tRNA may be ^{14}C-glutamic acid derived from ^{14}C-glutamine. E. coli extracts do not catalyze acceptance of glutamic acid by glutamine-specific tRNA (12). To test whether the ^{14}C-PCA-tRNA formed results from the GCT-catalyzed cyclization of ^{14}C-glutaminyl- or ^{14}C-glutamyl-tRNA, the aminoacyl-tRNA was incubated with the enzyme, isolated by ethanol precipitation and deacylated. The neutralized deacylation mixtures were subjected to paper electrophoresis and the electropherograms counted. The results shown in Figure 1 demonstrate that nearly half of the radioactivity recovered from the ^{14}C-aminoacyl-tRNA preparation is ^{14}C-glutamic acid. The proportion of ^{14}C-glutamate is identical in the presence or absence of the enzyme. In the presence of GCT (Figure 1B), however,

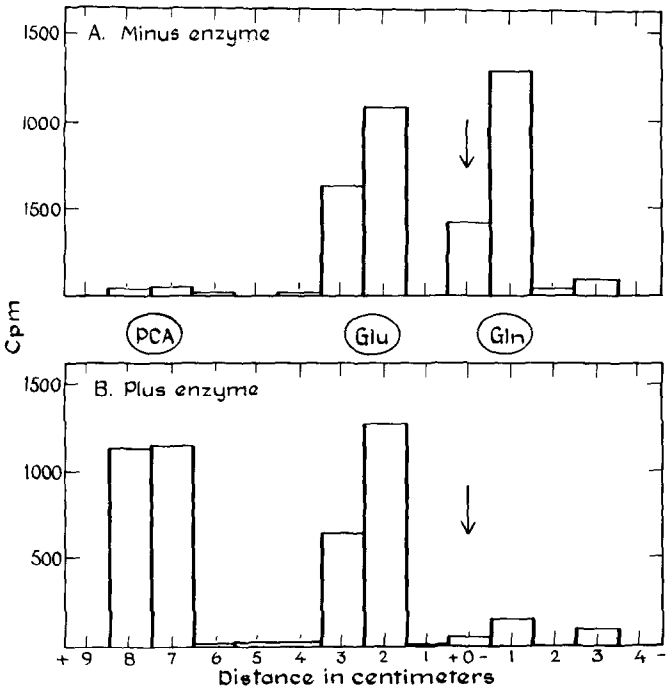

Figure 1. Paper electropherograms of products derived from the deacylation of ^{14}C-aminoacyl-tRNA isolated from reaction mixtures. Reaction mixtures and ethanol precipitation were as in Table IV. Incubation was for 30 minutes in the absence (A) and presence (B) of GCT. Electrophoretic mobility at pH 4.1 was determined by authentic markers run simultaneously. The arrows indicate the point of sample application.

TABLE I

Glutamine Cyclotransferase Action on Free Amino Acids

	Amino Acid and Time of Incubation					
	^{14}C-Glutamine		^{14}C-Glutamic acid		^{14}C-PCA	
	0 min	60 min	0 min	60 min	0 min	60 min
Electrophoretic Spot	Percentage of total radioactivity					
Glutamine	95.4	89.8	2.2	3.0	1.2	1.1
Glutamic acid	1.0	1.5	93.7	92.5	1.8	0.8
PCA	0.8	6.2	1.2	1.4	96.4	97.6
Total	97.2	97.5	97.1	96.9	99.4	99.5

Reaction mixtures contained 10^{-4} M amino acid (specific activities (μc/μmole); ^{14}C-glutamine, 4.45; ^{14}C-glutamic acid, 2.53; ^{14}C-PCA, 2.50) and were assayed by paper electrophoresis as described in Experimental. The figures represent the percentage of total radioactivity recovered on the electropherogram.

TABLE II

Glutamine Cyclotransferase Action on Free Amino Acids

	Amino Acid		
	^{14}C-Glutamine 35,700 cpm	^{14}C-Glutamic acid 34,100 cpm	^{14}C-PCA 24,000 cpm
	cpm recovered in effluent		
Minus Enzyme	428	1,756	19,553
Plus Enzyme	923	1,829	20,172

Reaction mixtures contained 10^{-4} M amino acid (as in Table I) and were assayed by the Dowex 50 column method as described in Experimental. The figures beneath the ^{14}C-amino acids indicate the radioactivity placed on the column and those in the table represent the radioactivity recovered in the effluent.

TABLE III

Glutamine Cyclotransferase Action on ^{14}C-Glutaminyl-tRNA

	Time of Incubation		
	0 min	30 min	30 min
	cpm recovered in effluent		TCA-precipitable cpm
Minus Enzyme	108	129	10,836
Plus Enzyme	918	6330	10,352

Reaction mixtures contained 85 A^{260}/ml tRNA (equivalent to 5.7×10^{-6} M ^{14}C-aminoacyl-tRNA, specific activity 41.3 μc/μmole) and were assayed by TCA precipitation or by the Dowex 50 column method described in Experimental. The figures represent the average of duplicate determinations.

TABLE IV

Glutamine Cyclotransferase Action on ^{14}C-Glutaminyl-tRNA

Ethanol precipitation after incubation

	Time of Incubation	
	0 min	30 min
	cpm recovered in effluent	
Minus Enzyme	159	138
Plus Enzyme	819	3834

Reaction mixtures were as described in Table III. After incubation, 0.25 mg carrier tRNA, 0.1 volume 2.0 M K acetate (pH 5.0) and 2.5 volume 95% ethanol were added and the mixture placed at -20°. The resultant precipitate was dissolved in water and assayed as in Table III. The figures represent the average of duplicate determinations.

the PCA recovered is totally accounted for by the disappearance of glutamine. Paper electrophoresis of an alkali deacylated, but otherwise untreated ^{14}C-aminoacyl-tRNA preparation yielded 40% ^{14}C-glutamate. Consequently, the substrate for the enzymatic formation of PCA-tRNA is glutaminyl-tRNA and the enzyme is without effect on glutamyl-tRNA.

DISCUSSION. Although PCA has been repeatedly shown to be an NH_2-terminal amino acid (e.g. B chain of fibrinogen (13), gastrin (14), light (15) and heavy (16) immunoglobulin chains and α_2 chain of collagen (17)), it has not been demonstrated to be incorporated into protein (3,4). Moav and Harris (4) have suggested that the source of PCA in immunoglobulins is not free PCA, but PCA-tRNA derived from a specific glutamyl-tRNA.

The studies reported here do not clarify whether NH_2-terminal PCA is formed at the polypeptide or tRNA level. However, these data demonstrate the *in vitro* formation of PCA-tRNA and suggest that the precursor may be glutaminyl-tRNA. The possibility that NH_2-terminal PCA arises from the cyclization of a specific glutaminyl-tRNA has precedence in the formation of N-formyl methionyl-tRNA (18). PCA and N-formyl methionine occur in proteins exclusively at the NH_2-terminal and neither compound has a free α-amino group which can participate in peptide bond formation. Further studies, particularly of the distribution of enzymes with similar activity, may show whether the PCA-tRNA complex is of any metabolic significance.

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