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THE OCCURRENCE OF γ-CARBOXYGLUTAMIC ACID IN MAMMALIAN RIBOSOMES

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SUMMARY. γ-carboxyglutamic acid (GLA) has been identified and estimated in mammalian ribosomes (mouse, rat, man, cultured cells) by amino acid analysis of total extracted protein. Assay results varied from 5.7-19.1 nMoles GLA/mg protein (6.0-9.9 residues GLA/1000 residues GLU), depending on purity and origin of ribosomes. GLA was not eliminated by purification. GLA in mouse hepatoma ribosomes was increased 3-fold upon preparation of puromycin-dissociated sub-units and sedimentation on gradients containing 1.0 M KCl. This tightly bound GLA (17.7 nMoles/mg sub-unit protein) could be present in one or more ribosomal proteins incorporated in the nucleoli, or in GLA-containing proteins acquired in the cytoplasm.

Warfarin has been shown to inhibit the K-dependent post-translational γ-carboxylation of glutamic acid residues in prothrombin (1). It has therefore been suggested that analogous reactions, such as those which produce the γ-carboxyglutamic acid (GLA) recently discovered in bone protein (2,3), may also be warfarin-sensitive (2). Inasmuch as we have shown that warfarin inhibits the *in vivo* synthesis of ribosomal RNA in mouse hepatoma (4), there was reason to suspect the participation of a GLA-containing protein in ribosomal RNA metabolism. If there is a warfarin-sensitive GLA-protein whose absence or deficiency of GLA can impair the synthesis of ribosomal RNA, it could well be among the ribosomal proteins. Defective structure or reduced availability of a single ribosomal protein would be likely to promote degradation of 45S precursor rRNA (5), thereby decreasing the rate at which mature rRNA can be produced. This could explain the inhibitory role of warfarin. Moreover, the quantity of GLA in ribosomal proteins might be a cellular means of controlling ribosome synthesis, and the presence of GLA in ribosomes might relate to their Mg^{++}- dependent functions.

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METHODS

Ribosomes were isolated by the method of Martin and Wool (6) from: tumors and livers from male C57L/J mice with BW 7756 hepatoma implants (Jackson Laboratories); brains from normal male Sprague-Dawley rats; SV-40 transformed human fibroblasts (SV80 cells, Massachusetts Institute of Technology Cell Culture Center); and human adenocarcinoma (obtained at craniotomy, Colorado General Hospital). Adenocarcinoma ribosomes were resuspended in Buffer A (0.1 M KCl, 1mM $MgCl_2$, 50mM TRIS·HCl, pH 7.3 at 5^0), clarified by centrifugation (10,000 x g, 10 min), layered over 4 ml 0.5M sucrose containing 1.0 M KCl, and centrifuged in Spinco type 50 rotor, 5^0, 6 hrs at 47,000 RPM. Hepatoma ribosomes (Experiment 5) were sedimented on gradients (15-30% sucrose in Buffer A, pH 7.8 at 25^0) in Spinco SW-41 rotor, 25^0, 4 hrs at 25,000 RPM. Hepatoma ribosomal sub-units (Experiment 6) were prepared from 20g tissue essentially as described by Sherton and Wool (7), except that sedimentation was on 36 gradients (15-30% sucrose, 1.0 M KCl, 10mM $MgCl_2$, 50mM TRIS·HCl, pH 7.8 at 25^0) in 6 centrifugations (Spinco SW-41 rotor, 25^0, 10 hrs at 22,500 RPM). Fractions containing 40S and 60S sub-units were combined, precipitated by 2/3 vol cold ethanol (8), and collected by centrifugation in Spinco type 30 rotor, 5^0, for 20 min at 20,000 RPM. Protein in ribosomes, sub-units, microsomes, and post-microsomal supernatants was separated from RNA by the LiCl-Urea method of Leboy, et al (9), precipitated by cold 10% TCA (1) in large volume to minimize salts and urea, washed with cold ethanol, and stored dry at -20^0 until analysis.

GLA was assayed as described by Hauschka (2) with minor modifications. Alkaline hydrolysis was in pyrex culture tubes with teflon-lined caps, using 0.3-0.5ml 2 N KOH. The hydrolysates, containing a few silica particles, were shaken twice with 4 ml H_2O and centrifuged before neutralization to pH 6.9-7.1 with dilute HCL. Samples were flash evaportated at 20^0, dissolved in citrate buffer, pH 2.2, and applied to a 0.9 x 54 cm column of AA-15 resin in a Beckman Model 120 C amino acid analyzer. Protein in the same solution was determined by the Lowry method (10). Chromatograms were recorded at 10-fold scale expansion for 90 min. GLA elutes at 20 min and GLU at 58 min. The GLA peak is easily distinguished, and is destroyed by heating the neutralized hydrolysate with 0.05 N HCl, as in (2). The elution time of GLA was established with 99.2% pure synthetic γ-carboxyglutamic acid (gift from Dr. Stuart G. Gordon). The same compound was used for determination of the ninhydrin color factor for GLA, as recommended by Hauschka (11). It was found to be 35.2% of the factor for GLU.

RESULTS AND DISCUSSION

Evidence that γ-carboxyglutamic acid is present in ribosomes has been obtained by amino acid analyses of proteins isolated from the ribosomes of 3 mammalian species, as shown in Table I. All results (except Experiment 1) were corroborated by a second amino acid analysis, performed after acid hydrolysis of the same material. This invariably destroyed, hence confirmed, the GLA peaks initially observed (2,11).

The occurrence of GLA in ribosomes, as ordinarily isolated, is illustrated by the results of Experiment 1. Preparation of the rat brain ribosomes (6g tissue), extraction of proteins, and analysis for GLA were as

Table 1. Determination of γ-carboxyglutamic acid in mammalian ribosomes

Samples (1-6 mg) of protein from ribosomes, ribosomal sub-units, microsomes, or postmicrosomal supernatants were hydrolyzed in 2 N KOH (22 hrs, 105°), diluted in H_2O, neutralized, and a portion heated with 0.05 N HCl (1.5 hrs, 100°) to destroy GLA (applies to all results except Experiment 1). Hydrolysates (+ HCl treatment) were flash evaporated and dissolved in 1.5 ml citrate, pH 2.2. 250 ul (55-432 ug protein) were applied to the column of an amino acid analyzer, and GLA was chromatographed in the amounts shown. The same chromatogram was used for calculation of residues GLA/1000 Residues GLU. The ninhydrin color factor was determined with synthetic GLA. See METHODS for details.

	ORIGIN OF PROTEIN	SAMPLE ANALYZED (ug)	GLA (nMoles)	GLA CONTENT (nMoles/ mg Protein)	RESIDUES GLA/ 1000 RESIDUES GLU
1	RAT BRAIN				
	Ribosomes	55	0.81	14.7	8.7
2	HUMAN ADENOCARCINOMA				
	Ribosomes (Purified)	80	1.29	16.1	9.5
3	HUMAN FIBROBLASTS				
	Microsomes	55	0.58	10.5	6.8
	Ribosomes	181	3.45	19.1	8.9
4	MOUSE LIVER				
	Ribosomes	329	2.14	6.5	7.9
	Postmicrosomal Supernatant	239	3.28	13.7	___
	MOUSE HEPATOMA				
	Ribosomes	351	2.00	5.7	6.0
	Postmicrosomal Supernatant	432	20.34	47.1	___
5	MOUSE HEPATOMA				
	Ribosomes (Partially Purified)	241	1.92	8.0	7.6
6	MOUSE HEPATOMA				
	Ribosomal Sub-units (Highly Purified)	330	5.84	17.7	9.9

described in METHODS. A significant level of GLA was found, as indicated, but there was doubt concerning the origin of this GLA. Since the ribosomes had not been rigorously purified, contamination by prothrombin or other foreign GLA proteins could not be ruled out. The likelihood of such contamination was greatly reduced in Experiment 2. These ribosomes, isolated immediately after excision of a 9.4g human adenocarcinoma, were resuspended and pelleted by centrifugation through a cushion of 0.5M sucrose, 1.0M KCl. It was expected that nearly all of the foreign proteins would be removed under these conditions (7,12), but assay of the purified tumor ribosomes showed that an appreciable complement of GLA was nevertheless retained.

Ribosomes from 8g of SV-40 transformed human fibroblasts (SV-80) were isolated in Experiment 3. They were not subjected to additional purification because contamination by extracellular GLA-proteins was extremely improbable. The only possible source of exogenous GLA would be the culture medium, and this was exhaustively washed from the cells before they were disrupted. The SV-80 ribosomes proved to contain much more GLA than was found in either of the preceding experiments. GLA was also assayed on a representative sample of the microsome pellets, before dissolution by detergent to free the ribosomes. By comparing both results in this experiment, it can be seen that the concentration of GLA was nearly 2-fold higher in the ribosomes than in the microsome pellet as a whole. This is clear evidence that GLA was localized in the ribosomes, or in proteins closely associated with them. But whichever is correct, it seems inescapable that GLA-proteins were synthesized in the SV-80 cells.

Ribosomes for Experiments 4-6 were prepared from the same mouse hepatoma in which we had found the synthesis of rRNA to be inhibited by Warfarin (4). Experiment 4 compares the GLA content of unpurified hepatoma and liver ribosomes. Also assayed in this experiment were the postmicrosomal supernatants, since they contain the foreign GLA-proteins to which the ribosomes were exposed during the lengthy isolation procedure. The results indicate that

a large difference in the GLA concentrations of the hepatoma and liver supernatants did not prevent the ribosomes of these tissues from being remarkably similar in GLA-content. Apparently the low level of GLA in these impure ribosomes was not determined by the availability of miscellaneous foreign GLA-proteins. However, this does not exclude the binding of specific GLA-proteins in small amounts. To determine whether or not the small parcel of GLA could be easily removed, the hepatoma ribosomes in Experiment 5 were sedimented on sucrose gradients containing 0.1M KCl. This low concentration of KCl does not remove tightly bound impurities from the ribosomes, nor does it foster dissociation into sub-units (13). Material sedimenting at less than 40S was discarded and the contents of all other fractions were recovered for GLA analysis, with results as shown. If compared with the results for impure hepatoma ribosomes (Experiment 4), it is seen that removal of freely soluble impurities did not lower the GLA content, but increased it significantly. Thus the GLA encountered in hepatoma ribosomes was not due to loosely bound impurities. Unless an integral part of the ribosome, this GLA could also have been present in tightly bound GLA-proteins, nascent polypeptides, or even in factors for protein synthesis.

All, or virtually all, of these possible sources of GLA can be eliminated by puromycin dissociation of the ribosomes and sedimentation of the sub-units on sucrose gradients containing 1.0M KCl (7,12). Hepatoma ribosomal sub-units were prepared in this manner for Experiment 6. The ratios of absorbance at 260:280nm and 260:235nm were 1.98 and 1.67, respectively, indicating a high degree of purity (13, 14). The GLA content of these highly purified sub-units was found to be 17.7 nMoles/mg protein, which is 3-fold higher than in unpurified hepatoma ribosomes (Experiment 4). Furthermore the identification and estimation of a significant quantity of GLA in purified ribosomal sub-units demonstrates that this unique amino acid is firmly associated with the ribosomal proteins.

In view of all the results presented it seems justified to postulate that

GLA-containing protein is a normal component of mammalian ribosomes. However, we do not yet have evidence that such GLA-protein is necessarily ribosomal, in the sense that it is incorporated together with rRNA during ribosomal assembly.

Based upon the data in Table I, ribosomes appear to contain very little GLA relative to protein. But the assays were performed on total ribosomal protein, comprising some 70 individual molecules (7). Taking the GLA content of ribosomes to be 17.7 nMoles/mg protein (Expt 6), and the "molecular weight" of total ribosomal protein to be 2.15×10^6 (12), it can be calculated that 1 mole of ribosomal protein contains 38 moles of GLA. Should all the GLA be on one of the 70 molecules (av Mol Wt 28,000), it could easily contain well over 100 residues GLA/1000 residues amino acid. This may be compared with Hauschka's purified bone protein, osteocalcin, which has 60 residues GLA/1000 residues amino acid (11). Therefore the GLA that we have found to be associated with ribosomes is ample to provide for one or more ribosomal proteins that are rich in GLA.

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