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γ-CARBOXYGLUTAMIC ACID IN INVERTEBRATES: ITS IDENTIFICATION IN HERMATYPIC CORALS

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Summary: The presence of a γ-carboxyglutamate-containing protein in hermatypic corals has been established. γ-Carboxyglutamate has been isolated from the alkaline hydrolysate of protein extracted from the coral *Lobophyllia corymbosa*, by chromatography of the hydrolysate on Dowex AG 1-X8 (formate form), followed by chromatography on an amino acid analyzer column. This procedure achieves complete separation of γ-carboxyglutamate from an acid-stable compound which is also present in the alkaline hydrolysate of coral protein, and which has proved difficult to separate from γ-carboxyglutamate by other methods. The identity of the γ-carboxyglutamate thus isolated has been established unequivocally by determining the yield of glutamic acid after acid treatment (2 M HCl, 6 h, 110°C). The color factor for the γ-carboxyglutamate isolated from *Lobophyllia* is 0.45_4 times the value for glutamic acid, in good agreement with the value determined for authentic γ-carboxyglutamic acid (0.45_8) under exactly the same conditions. Virtually identical results have been obtained for the coral, *Acropora cuneata*. These experiments provide the first secure evidence for the presence of γ-carboxyglutamate in an invertebrate species, and they clearly have important implications for our understanding of invertebrate mineralization.

In 1974, three laboratories described a new amino acid, γ-carboxyglutamic acid (Gla)[1] in the blood-clotting protein prothrombin (1-3). Gla was shown to be responsible for the calcium-binding properties of prothrombin (1-4) and to be synthesized by the post-translational, vitamin K_1 dependent carboxylation of specific Glu residues in preprothrombin (5). The rapid development of this area of vitamin K_1 dependent carboxylations led to the important discovery that Gla-containing proteins distinct from the blood-clotting proteins were ubiquitous constituents of vertebrate mineralized tissue. Gla-containing proteins [osteocalcins (6) or bone Gla proteins (7)] have been isolated from a number of sources, and they appear to be highly homologous (8).

The broad distribution of Gla-containing proteins in vertebrate mineralized tissue leaves little doubt that such proteins have an essential role in the process of mineralization. It is surprising, therefore, that all attempts to demonstrate the presence of Gla in an invertebrate species have so far been unsuccessful. King (9) found no Gla in foraminifera, mollusc shell, sea urchin spine, crab carapace or brachiopod shell, and Price (7) failed to detect Gla in the calcified cartilage of elasmobranchs. Taken at face value, these results would suggest that there exist essentially different mechanisms of mineralization in vertebrate and invertebrate species. However, a clear reason for the failure of previous workers to detect Gla in invertebrate species is to be found in their choice of mineralized tissues containing little protein *of any kind.* We have therefore examined two species of hermatypic coral for the presence of Gla. Our results are reported in this Communication.

[1] Abbreviations: Gla, γ-carboxyglutamic acid; Asa, β-carboxyaspartic acid.

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Experimental Section

Hermatypic corals, *Acropora cuneata* (Verill) and *Lobophyllia corymbosa* (Forskaal), from the reef flat at Heron Island, southern Great Barrier Reef, were obtained through the courtesy of the Heron Island Research Station, and were stored at −20°C. The coral was crushed, demineralized with 20% (w/v) formic acid (~3 ml/g of tissue), and desalted on Sephadex G-25 at 4°C as described by Poser *et al.* (8). The extracted protein was lyophilized and partially purified by chromatography on a column (87 X 5.7 cm) of Sephadex G-100 equilibrated with 0.05 M ammonium bicarbonate. Fractions eluted between 2 and 4.5 void volumes were pooled and lyophilized. Gla was analyzed as follows: protein samples were hydrolyzed (2 M KOH, 22 h, 110°C) and then desalted by the method of Fernlund *et al.* (10). Amino acid analyses were performed on a Technicon Amino Acid Analyzer (TSM), using an elution program adapted from the procedure of Hauschka (6). A column (28 X 0.5 cm) of Technicon Chromobeads Type C-3 was equilibrated at 60°C at a flow rate of 15 ml/h. All buffers contained 0.2 M citrate, 0.23% (w/w) Brij and 0.1% (w/v) phenol. The sodium ion concentration was 0.16 M at pH 2.68 and pH 3.10, and 0.2 M at pH 3.44. The following buffer program was used: pH 2.68, 4 min; pH 3.10, 15 min; pH 3.44, 60 min; 0.2 M NaOH, 16 min; pH 2.68, 28 min. Standards were eluted at the following times: cysteic acid, 8.5 min; Asa, 11 min; Gla, 31.5 min; Asp, 51 min; Glu, 60 min. Gla was synthesized as described by Fernlund *et al.* (10). The color factor for Gla, determined in independent trials by peak area determination before and after acid treatment (2 M HCl, 6 h, 110°C) was $0.45_8 \pm 0.03_8$ (2 σ) times the value for Glu [*cf.* 0.42_0 (6)]. Because the elution time of Gla is remarkably sensitive to the presence of salts, solvents and coeluting compounds, any putative Gla peak was always assessed by analysis of an independent sample to which authentic Gla had been added. Asa was kindly supplied by Professor Alan Sargeson (11). The color factor for Asa, determined as described for Gla, was $0.79_3 \pm 0.18$ (2 σ) times the value for Asp [*cf.* 0.78_1 (12)].

The highly acidic components present in alkaline hydrolysates of coral protein were isolated by chromatography on a column (30 X 1.2 cm) of Dowex AG 1-X8 at a flow rate of 1.2 ml/min at 25°C. The hydrolysate (~1.5 ml) was applied to the column which was washed with distilled water (200 ml) to remove neutral and basic amino acids. A stepwise pH gradient of aqueous formic acid solutions was applied to elute the highly acidic components: pH 2.50, 220 ml; pH 2.28, 100 ml; pH 2.07, 240 ml; pH 1.46, 110 ml; and 0.1 M HCl, 240 ml. Standard amino acids were eluted as follows – Asp: pH 2.50, 100-120 ml; Gla: pH 2.07, 126-181 ml; Asa: pH 1.46, 65-85 ml. Fractions which were eluted during the chromatography of coral hydrolysates were pooled accordingly, then lyophilized and reanalyzed. If necessary, these fractions were further purified by chromatography on the amino acid analyzer column using the buffer elution program employed for routine analysis (*vide supra*). The purified highly acidic components were treated with acid (2 M HCl, 6 h, 110°C), dried *in vacuo* and reanalyzed. Proline was added as an internal standard prior to acid treatment.

Results and Discussion

Alkaline hydrolysates of the *crude* formic-acid extracted protein from *Lobophyllia* and *Acropora* contain at least two ninhydrin-positive species which coelute with authentic Gla on amino acid analysis. Acid treatment of the alkaline hydrolysates of *crude* coral protein under conditions which cause complete decarboxylation of authentic Gla to Glu result in only partial loss (≤20%) of the material coeluting with Gla in the coral hydrolysates. Very similar results are obtained when the crude EDTA-extracted protein from human teeth is analyzed, except that the proportion (80-90%) of Gla is much larger (13). Attempts to separate the acid-labile compound in coral, tentatively identified as Gla, from the acid-stable species, using a modified buffer program on the amino acid analyzer, proved unsuccessful. However, the putative Gla was successfully isolated from alkaline hydrolysates of *Lobophyllia* protein by chromatography of the hydrolysate on a column of Dowex AG 1-X8 resin in the formate form. Two ninhydrin-positive compounds, A and B, were eluted in a position close to authentic Gla. Compound A, which was eluted within the first 70 ml of pH 2.07 formic acid, coeluted with Gla on amino acid analysis, and was relatively stable in acid. Compound B, which was eluted between 125 ml and 162 ml of pH 2.07 formic acid, also coeluted with Gla on amino acid analysis. Acid treatment of compound B caused the disappearance of not less than 95% of this material and the production of Glu (Fig. 1). The color factor for B was $0.45_4 \pm 0.04$ (2 σ) times that of Glu, identical to the value of $0.45_8 \pm 0.03_8$ (2 σ), determined for authentic Gla. Compound B is thus unequivocally identified as Gla. Virtually identical results were obtained when the formic-acid extracted protein from *Acropora* was treated as described above. In this case, however, the Dowex chromatography step failed to resolve Gla completely from the acid-stable component. Acid treatment of the mixture of putative Gla and the acid-stable component resulted in the loss of 48% of the total material and the production of Glu. The color factor for the acid-labile component was $0.42_0 \pm 0.03_8$ (2 σ) times that of Glu, again identifying it as Gla.

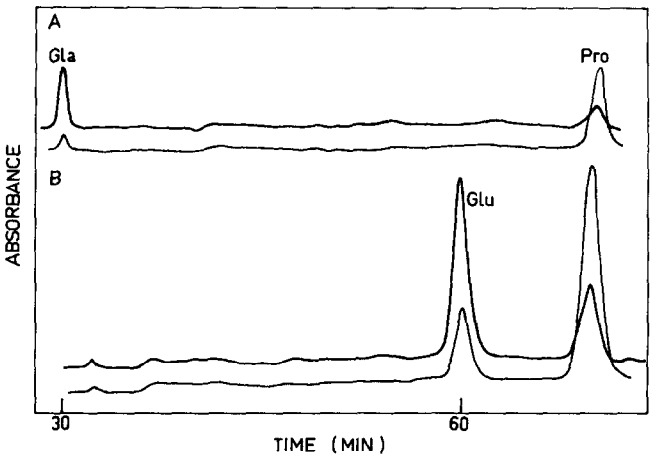

Figure 1. Untouched elution profiles from the amino acid analyzer of Gla isolated from the alkaline hydrolysate of *Lobophyllia* protein. A, before treatment with acid; B, after treatment with acid. The sample size in B is three times that in A. Proline was added to the isolated Gla as an internal standard prior to acid treatment. Note the variability in elution time of the ~31 min peaks. Both peaks coelute with authentic Gla.

TABLE I

Levels of Gla in Hermatypic Corals, Vertebrate Mineralized Tissues and Pure Proteins

Tissue	[Gla] nmol/g of tissue	Gla residues/1000 residues of Glu[a]
CHICKEN BONE[b]		
Whole bone	–	2-8
EDTA extract	–	100-136
G-100 fraction	–	424
Pure osteocalcin	–	550
FETAL CALF MOLAR[b]		
EDTA extract	–	5-23
STEER TEETH[c]		
Dentine	260	–
CALF BONE[c]		
Whole bone	1,580	<10
EDTA extract	1,110	160
G-100 fraction	1,070	510
Pure bone Gla protein[d]	–	1,000
REEF-BUILDING CORALS[e]		
Acropora	~1	~4
Lobophyllia	~1	⩽3
G-100 fraction (*L.*)	–	19

[a]Determined for alkaline hydrolysates. [b]Reference 6. [c]Reference 7. [d]Reference 8. [e]Present work.

Table I provides a comparison of the approximate levels of Gla in *Lobophyllia* and *Acropora* with the levels present in some vertebrate mineralized tissues. A comparison of columns two and three of Table I demonstrates that the yield of Gla in coral (nmol/g of tissue) is *not* due to an inherently different Gla-containing protein, but rather that the major material analyzed is calcium carbonate.

In 1977, Hauschka reported the occurrence of a "peak" eluting 2 minutes earlier than independently analyzed Gla. This material was present in a variety of tissues including molluscan shell proteins. He also reported that it was stable to acid hydrolysis. The present work points up two facts: (i) the elution time of Gla is remarkably sensitive to the presence of salts, solvents and coeluting compounds; (ii) casual inspection of the results of analyses for acid stability of fractions may fail to detect small amounts of Gla (or other acid-labile amino acids) which may be present, and whose elution may be affected as in (i).

It is now reasonable that Gla may be of fundamental significance in the mineralization of tissues, both vertebrate and invertebrate. For this reason, we are currently extending this work to include corals of the order ALCYONACEA, the "soft corals", many of which exhibit very limited tissue mineralization. If the Gla-containing protein(s) of hermatypic corals have an essential role in mineralization, then the Gla levels in hard and soft corals may be expected to reflect this. In this connection, it may be relevant that Hauschka *et al.* failed to report the presence of Gla in unspecified branched soft corals (12).

Moreover, we have recently reported the carboxylation of t-butyloxycarbonylaspartic acid α-benzyl ester by a carboxylase which is both vitamin K_1 and pyridoxal phosphate dependent (14). A related search for Asa [as yet unequivocally identified only in *E. coli* ribosomal proteins (15)] continues under active investigation (13).

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References

1. Stenflo, J., Fernlund, P., Egan, W., and Roepstorff, P. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2730-2733.
2. Nelsestuen, G.L., Zytovicz, T.H., and Howard, J.B. (1974) *J. Biol. Chem.* **249**, 6347-6350.
3. Magnusson, S., Sotrupp-Jensen, L., Petersen, T.E., Morris, H.R., and Dell, A. (1974) *F.E.B.S. Lett.* **44**, 189-193.
4. Nelsestuen, G.L., and Suttie, J.W. (1972) *Biochemistry* **11**, 4961-4964.
5. Esmon, C.T., Sadowski, J.A., and Suttie, J.W. (1975) *J. Biol. Chem.* **250**, 4744-4748.
6. Hauschka, P.V. (1977) *Anal. Biochem.* **80**, 212-223.
7. Price, P.A., Otsuka, A.S., Poser, J.W., Kristaponis, J., and Raman, N. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1447-1451.
8. Poser, J.W., Esch, F.S., Ling, N.C., and Price, P.A. (1980) *J. Biol. Chem.* **255**, 8685-8691.
9. King, K. Jr (1978) *Biochim. Biophys. Acta* **542**, 542-546.
10. Fernlund, P., Stenflo, J., Roepstorff, P., and Thomsen, J. (1975) *J. Biol. Chem.* **250**, 6125-6133.
11. Dixon, N.E., and Sargeson, A.M. (1982) *J. Amer. Chem. Soc.*, in press.
12. Hauschka, P.V., Henson, E.B., and Gallop, P.M. (1980) *Anal. Biochem.* **108**, 57-63.
13. Zerner, B. (1979) *Proc. Aust. Biochem. Soc.* P2.
14. Hamilton, S.E., Tesch, D., and Zerner, B. (1982) *Biochem. Biophys. Res. Commun.*, in press.
15. Christy, M.R., Barkley, R.M., Koch, T.H., Van Buskirk, J.J., and Kirsch, W.M. (1981) *J. Amer. Chem. Soc.* **103**, 3935-3937.