Gallop, P. M. (1955), *Arch. Biochem. Biophys. 54*, 486.
Gallop, P. M., Seifter, S., and Meilman, E. (1957a), *J. Biophys. Biochem. Cytol. 3*, 545.
Gallop, P. M., Seifter, S., and Meilman, E. (1957b), *J. Biol. Chem. 227*, 891.
Gellert, M. F., von Hippel, P. H., Schachman, H. K., and Morales, M. F. (1959), *J. Am. Chem. Soc. 81*, 1384.
Gordon, R. S., Jr. (1948), cited in Ferry (1948), p. 25.
Green, A. A. (1931), *J. Biol. Chem. 93*, 495.
Gustavson, K. H. (1956), *in* The Chemistry and Reactivity of Collagen, New York, Academic Press, Inc.
Harrington, W. F. (1958), *Nature 181*, 997.
Harrington, W. F., and Sela, M. (1958), *Biochim. et Biophys. Acta 27*, 24.
Harrington, W. F., and von Hippel, P. H. (1961a), *Advances in Protein Chem. 16*, 1.
Harrington, W. F., and von Hippel, P. H. (1961b), *Arch. Biochem. Biophys. 92*, 100.
Katz, J. R., and Wienhoven, J. F. (1933), *Rec. trav. chim. 52*, 36.
Long, F. A., and McDevit, W. F. (1952), *Chem. Revs. 51*, 119.
Piez, K. A. (1960), *J. Am. Chem. Soc. 82*, 247.
Piez, K. A., and Gross, J. (1960), *J. Biol. Chem. 235*, 995.
Pleass, W. B. (1930), *Proc. Roy. London, A 126*, 406.
Ramachandran, G. N. (1956), *Nature 177*, 710.
Rich, A., and Crick, F. H. C. (1955), *Nature 176*, 915.
Rich, A., and Crick, F. H. C. (1961), *J. Mol. Biol. 3*, 483.
Robinson, R. A., and Stokes, R. H. (1959), *in* Electrolyte Solutions, ed. 2, New York, Academic Press, Inc.
Seifter, S., Gallop, P. M., Klein, L., and Meilman, E. (1959), *J. Biol. Chem. 234*, 285.
Steinberg, I. Z., Harrington, W. F., Berger, A., Sela, M., and Katchalski, E. (1960), *J. Am. Chem. Soc. 82*, 5263.
von Hippel, P. H., Gallop, P. M., Seifter, S., and Cunningham, R. S. (1960), *J. Am. Chem. Soc. 82*, 2774.
von Hippel, P. H., and Harrington, W. F. (1959), *Biochim. et Biophys. Acta 36*, 427.
von Hippel, P. H., and Harrington, W. F. (1960), *in* Protein Structure and Function, *Brookhaven Symposia in Biol. 13*, 213.

Polymerization Reactions Catalyzed by Intracellular Proteinases. III. Action of Cathepsin C on a Tetrapeptide Amide*

JOSEPH S. FRUTON AND MARGARET H. KNAPPENBERGER

From the Department of Biochemistry, Yale University, New Haven, Connecticut

Received February 21, 1962

When cathepsin C acts at *p*H 7.5 on glycyl-L-tyrosylglycyl-L-tyrosinamide labeled with C^{14} in one of the two glycine residues, the labeling of the resulting polymeric peptide amide indicates that both dipeptide units are incorporated, but only after prior cleavage of the interior tyrosylglycyl bond. The data are in accord with the hypothesis that the tetrapeptide amide is an intermediate in the enzymic polymerization of glycyl-L-tyrosinamide only when the tetrapeptide amide is formed and held by the enzyme.

In previous communications from this laboratory, it was reported that at *p*H 7.5, cathepsin C converts glycyl-L-tyrosinamide to a decapeptide amide (Fruton *et al.*, 1953), and that during this polymerization reaction glycyl-L-tyrosylglycyl-L-tyrosinamide appears in the incubation mixture (Würz *et al.*, 1962). Experiments designed to determine the role of the tetrapeptide amide in polymer formation indicated that this compound, when added to the incubation mixture, did not serve as a "primer" in the polymerization of glycyl-L-tyrosinamide. When synthetic tetrapeptide amide was present in an incubation mixture containing C^{14}-labeled glycyl-L-tyrosinamide, the radioactivity of the resulting polymer was reduced, as compared with a control experiment in the absence of tetrapeptide amide, indicating that the unlabeled tetrapeptide amide had contributed glycyltyrosyl units to the formation of the polymer. Furthermore, cathepsin C was shown to act on glycyl-L-tyrosylglycyl-L-tyrosinamide at *p*H 7.5 with the formation of glycyl-L-tyrosine and of polymer, and with slow evolution of ammonia, indicating a primary attack of the enzyme at the interior tyrosylglycyl peptide bond. The available data led to the suggestion that in the polymerization of glycyl-L-tyrosinamide by cathepsin C, the tetrapeptide amide only serves as an intermediate when it remains bound to the catalytic region of the enzyme; if released from the site of its formation, or if added to the incubation mixture, the tetrapeptide amide appears to be cleaved to dipeptide units (possibly in the form of glycyltyrosyl-enzyme) before re-utilization for polymer formation.

In the present communication, further data are presented in support of the conclusion that free glycyl-L-tyrosylglycyl-L-tyrosinamide, when acted upon by cathepsin C, is cleaved before utilization for polymer formation.

---

* This work was aided by grants from the U. S. Public Health Service (RG-6452) and the National Science Foundation (G-7451).

EXPERIMENTAL

The cathepsin C preparation was the same as that used in the experiments of Würz *et al.* (1962), and the enzyme concentration was 0.4 units per ml of incubation mixture. The enzyme experiments were conducted at 37.5° and *p*H 7.5–7.6 (adjusted with 0.1 N NaOH, the peptide amides serving as buffers), with 0.01 M β-mercaptoethylamine as the enzyme activator.

Two samples of glycyl-L-tyrosylglycyl-L-tyrosinamide acetate were used in these experiments. Both were synthesized by the method described by Würz *et al.* (1962). One of them was made by coupling C^{14}-labeled carbobenzoxyglycyl-L-tyrosine (made with uniformly labeled C^{14}-glycine, 3260 cpm per μmole) with glycyl-L-tyrosine ethyl ester hydrochloride to yield as the final product the tetrapeptide amide acetate (N, 13.4; for the carbobenzoxytetrapeptide amide, m.p. 220–221° decomp.; N, 11.7) labeled in the amino-terminal glycyl residue, with a specific radioactivity of 1600 cpm per μmole tyrosine. This sample of tetrapeptide amide will be designated G*TGTA. The other sample of synthetic tetrapeptide amide acetate was made from carbobenzoxyglycyl-L-tyrosine and C^{14}-labeled glycyl-L-tyrosine ethyl ester hydrochloride (3260 cpm per μmole, labeled in the glycyl group) to yield a product (N, 13.6; for the carbobenzoxytetrapeptide amide, m.p. 221–222° decomp.; N, 11.6), with a specific radioactivity of 1610 cpm per μmole tyrosine. This sample will be designated GTG*TA. The specific radioactivity of G*TGTA and GTG*TA is given in cpm per μmole tyrosine because a spectrophotometric method is used for the determination of the concentration of polymer solutions (for details, see Würz *et al.*, 1962). The specificity of cathepsin C, which is restricted in its action at peptide bonds two α-amino acid residues away from a free α-amino group (Izumiya and Fruton, 1956; Planta and Gruber, 1961), permits one to treat the glycyltyrosyl group as a unit for purposes of determination of specific radioactivity. The validity of this procedure is supported by the concordance of the data presented in this communication with those obtained by Würz *et al.* (1962) in experiments with glycyl-L-tyrosinamide labeled with C^{14} in the tyrosyl residue. The isolation of the polymer, the determination of its radioactivity, and the preparation of its dinitrophenyl (DNP) derivative, were performed in the manner described previously (Würz *et al.*, 1962). The DNP-polymer was hydrolyzed for 20 hours at 110° with 6 N HCl, and the resulting DNP-glycine was extracted with ether; an aliquot of the ether extract was plated and counted. The concentration of DNP-glycine was estimated spectrophotometrically, with a value of 15,000 (in glacial acetic acid) used for the molar extinction coefficient at 340 mμ.

RESULTS

The earlier results of Würz *et al.* (1962) had shown that when 0.05 M glycyl-L-tyrosinamide labeled with C^{14} in the tyrosine residue (4560 cpm per μmole) is incubated with cathepsin C in the presence of unlabeled 0.025 M glycyl-L-tyrosylglycyl-L-tyrosinamide acetate, the specific radioactivity of the resulting polymer was 1,840 cpm per μmole tyrosine; with 0.01 M tetrapeptide amide, the polymer had 3,120 cpm per μmole of tyrosine. In the absence of added tetrapeptide amide, the polymer had 4,650 cpm per μmole tyrosine. Therefore, the extent to which 0.025 M tetrapeptide amide had contributed glycyltyrosyl units to the polymer amounted to 60.4% (100 − [1,840 × 100/4,650]), and with 0.01 M tetrapeptide amide it was 33.2%. The finding that 0.025 M tetrapeptide amide (0.05 M with respect to glycyltyrosyl units) caused more than 50% dilution of the label suggested that the tetrapeptide amide is a somewhat better donor of glycyltyrosyl units than is the dipeptide amide.

To examine this question further, the enzymic formation of polymer was studied with unlabeled glycyl-L-tyrosinamide and labeled tetrapeptide amide (G*TGTA or GTG*TA). The results of a representative experiment are summarized in Table I, from which it will be seen that both G*TGTA and GTG*TA donated glycyltyrosyl units to the polymer. Although the specific radioactivity of the labeled glycine residue was the same in both samples of tetrapeptide amide (3200 cpm per μmole of the labeled glycine residue), equivalent concentrations of the two samples caused a different extent of labeling in the polymer. The results in Table I are consistent with the view that the amino-terminal glycyltyrosyl unit is utilized more effectively for polymer formation than the carboxyl-terminal dipeptide unit. Calculation of the extent to which G*TGTA and GTG*TA at 0.025 M contributed labeled glycyl-

TABLE I

ACTION OF CATHEPSIN C ON GLYCYL-L-TYROSINAMIDE IN THE PRESENCE OF C^{14}-LABELED TETRAPEPTIDE AMIDE

The reaction mixtures (1 ml) contained 50 μmoles of glycyl-L-tyrosinamide acetate and labeled glycyl-L-tyrosylglycyl-L-tyrosinamide acetate (10 μmoles or 25 μmoles). Incubation period, 2.5 hours.

Labeled Tetrapeptide Amide	Time of Appearance of Polymer (min.)	Yield of Polymer (μmoles tyrosine)	Specific Radioactivity of Polymer (cpm/μmole tyrosine)
G*TGTA (10 μmoles)	28	13.7	590
G*TGTA (25 μmoles)	42	24.1	1050
GTG*TA (10 μmoles)	28	13.0	445
GTG*TA (25 μmoles)	38	24.4	850

tyrosyl units to the polymer gives values of 32.8% and 26.6% respectively; the total of 59.4% may be compared with the value of 60.4% calculated for the contribution of unlabeled glycyltyrosyl units by the tetrapeptide amide (0.025 M) in the presence of C^{14}-labeled glycyl-L-tyrosinamide (0.05 M). A similar calculation for the extent of incorporation of labeled glycyltyrosyl units from G*TGTA and GTG*TA at 0.01 M gives values of 18.4% and 13.9% respectively; the total of 32.3% may be compared with the value of 33.2% obtained for the contribution of unlabeled glycyltyrosyl units by 0.01 M unlabeled tetrapeptide. The satisfactory agreement for the sets of data obtained in one case with glycyl-L-tyrosinamide labeled in the tyrosine residue, and in the other case with tetrapeptide amide samples labeled in one of the glycine residues, further supports the view that cathepsin C catalyzes the transfer of dipeptide units.

The values for the specific radioactivity given in Table I suggest that, in the presence of 0.05 M glycyl-L-tyrosinamide, the amino-terminal glycyltyrosyl unit of the tetrapeptide amide is utilized about 25% more effectively for polymer formation than is the other dipeptide unit of the tetrapeptide amide. The significant difference in the effectiveness of the two samples of labeled tetrapeptide amide in labeling the polymer may also be considered as additional (although not conclusive) evidence against the role of the tetrapeptide amide as a "primer" in the polymerization of glycyl-L-tyrosinamide. If the tetrapeptide amide served solely as a primer, the extent of labeling of the polymer should have been the same with both samples of labeled tetrapeptide amide.

It will be noted from Table I that the yield of polymer was greater in the experiments with 0.025 M tetrapeptide amide than with 0.01 M tetrapeptide amide, as further indication of the fact that the tetrapeptide amide donated glycyltyrosyl units to the polymer. The delay in the appearance of the insoluble polymer at the higher concentration of tetrapeptide amide was noted previously (Würz *et al.*, 1962), but no explanation for this increased lag can be offered at present.

The results obtained are consistent with the conclusion that cathepsin C catalyzed the reaction:

$$\text{GTGTA} + \text{Enzyme} \rightleftharpoons \text{GT-Enzyme} + \text{GTA}$$

In the presence of a high concentration of a suitable acceptor such as glycyl-L-tyrosinamide (GTA), GT-enzyme may be assumed to contribute GT units to the growing polymer chain. In the absence of glycyl-L-tyrosinamide, polymer formation is observed but the time of its appearance is much delayed (Table II), presumably because of the low initial concentration of the dipeptide amide (GTA) and the apparent inability of the tetrapeptide amide to serve as an effective acceptor of glycyltyrosyl units in the transamidation reaction. It may be expected, however, that as the reaction proceeds and the dipeptide amide increases in concentration, polymerization will occur with the incorporation of both dipeptide units of the tetrapeptide amide. The data presented in Table II are in accord with this expectation. The polymer obtained from G*TGTA was somewhat more highly labeled than that from GTG*TA, although the specific radioactivity of the two tetrapeptides was the same, again indicating preferential utilization of the amino-terminal dipeptide unit for polymer formation. Although the chain length of the polymers obtained in these experiments was not determined, it may be assumed from earlier work that they are (on the average) decapeptide amides. On this basis, the value of 1860 cpm per μmole tyrosine for the polymer from G*TGTA (Table II) would correspond to an average ratio of labeled to unlabeled glycine in the polymer of about 3:2 (theory, 3200 × 3/5 = 1920), and the value of 1320 for the polymer from GTG*TA would correspond to an average ratio of about 2:3 (theory, 3200 × 2/5 = 1280).

TABLE II

ACTION OF CATHEPSIN C ON C^{14}-LABELED GLYCYL-L-TYROSYLGLYCYL-L-TYROSINAMIDE ACETATE

The reaction mixtures (1 ml) contained 50 μmoles of labeled tetrapeptide amide. Incubation period, 4 hours. The polymer appeared within 2.5 hours in both experiments.

Labeled Tetrapeptide Amide	Yield of Polymer (μmoles tyrosine)	Specific Radioactivity of Polymer (cpm/μmole tyrosine)
G*TGTA	15.8	1860
GTG*TA	15.0	1320

The suggestion that the dipeptide units of the labeled tetrapeptide are utilized in the polymerization reaction independently of each other is further supported by the observation that the polymer derived from GTG*TA contained peptide chains with C^{14}-labeled amino-terminal glycine residues. The specific radioactivity (880 cpm per μmole) of the DNP-glycine obtained on hydrolysis of the DNP-substituted polymer from GTG*TA suggests that approximately 27% (880 × 100/3200) of the peptide chains contained labeled amino-terminal glycine residues.

REFERENCES

Fruton, J. S., Hearn, W. R., Ingram, V. M., Wiggans, D. S., and Winitz, M. (1953), *J. Biol. Chem. 204*, 891.

Izumiya, N., and Fruton, J. S. (1956), *J. Biol. Chem. 218*, 59.

Planta, R. J., and Gruber, M. (1961), *Biochim. et Biophys. Acta 53*, 443.

Würz, H., Tanaka, A., and Fruton, J. S. (1962), *Biochemistry 1*, 19.