# CATALYTIC FORMATION OF ε-(γ-GLUTAMYL)LYSINE IN GUINEA PIG LIVER TRANSGLUTAMINASE

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SUMMARY

Incorporation of putrescine into purified guinea pig liver transglutaminase was observed when the enzyme was incubated under catalytic conditions in the absence of the exogenous acceptor substrate casein. Furthermore, in the absence of exogenous substrates, putrescine and casein, the enzyme monomer of 85-90,000 daltons was converted to polymeric material. ε(γ-Glutamyl)lysine isopeptide was isolated from polymeric transglutaminase samples following proteolytic digestion. The results were consistent with the formation of an intermolecular isopeptide, ε(γ-glutamyl)lysine, between two or more enzyme monomers apparently by an autocatalytic reaction.

## INTRODUCTION

Transglutaminase catalyzes the $Ca^{++}$-dependent acyl transfer reaction between peptide-bound γ-carboxamide glutamine moieties and various primary amines (1, 2). When the primary amine is the ε-amino group of protein-bound lysine, inter- or intramolecular ε-(γ-glutamyl)lysine isopeptides are produced. Materials containing such crosslinks usually possess a high degree of insolubility in physiological buffers (3-9). While transglutaminases have been demonstrated in numerous biological systems (see 2, 10 for reviews) a definite biological function has not been established for many of these materials.

In the course of studies on transglutaminase activity in normal and transformed cells, we observed significant putrescine incorporation into protein when the acceptor protein casein was omitted from the assay mixture





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(11, 12). While this observation was not unique (1), our efforts to completely separate amine acceptor from enzymatic activity were unsuccessful. We postulated that enzymatic activity and amine acceptors might reside on the same molecule (11, 12). We report here that guinea pig liver transglutaminase purified essentially to homogeneity shows putrescine incorporation into protein in the absence of an exogenous acceptor protein. Furthermore, the 85,000 dalton catalytic band disappeared and polypeptides with a molecular weight in excess of 250,000 daltons appeared when reaction mixtures deficient in putrescine and casein were examined upon sodium dodecylsulfate polyacrylamide gel electrophoresis. Isopeptide crosslink formation was confirmed by the isolation of ε-(γ-glutamyl)lysine from samples containing the high molecular weight material.

## MATERIALS AND METHODS

Guinea pig liver transglutaminase (8500 units/mg) was purified according to the procedure of Connellan et al. (13) and stored frozen at -20°. A 2 ml reaction mixture containing 1 mg purified transglutaminase, 10 mM Tris-HCl, pH 8.3, 10 mM dithiothreitol, and either 5 mM $CaCl_2$ or 1 mM $Na_2$ EDTA was incubated at 37° for 60 min. After incubation 0.1 ml aliquots were removed for electrophoresis while the remainder was placed in a boiling water bath for 1-2 min and then processed for isopeptide content.

Electrophoresis (0.5 x 6 cm gels, 5.6% in acrylamide, 1% sodium dodecylsulfate) was run for 65 min at a current of 8 mamps/gel according to the procedure of Fairbanks et al. (14). Molecular weight standards were purchased from Biodynamics/bmc, New York, NY, and included cytochrome c, chymotrypsinogen A, aldolase, catalase, and bovine albumin.

ε-(γ-Glutamyl)lysine isopeptide was determined after exhaustive enzyme digestion (8) of guinea pig liver transglutaminase by pronase (Calbiochem, La Jolla, CA) and leucine aminopeptidase (Worthington, Freehold, NJ). A known amount of [$^3$H] ε-(γ-glutamyl)lysine was added, the digest desalted (15), lyophilized, dissolved in 0.1 N hydrochloric acid, placed on a 0.6 x 10.5 cm column (HPB-80 resin, Hamilton Co., Reno, NV), and chromatographed at 50° C using a pyridine acetate buffer gradient (0.05 M, pH 3.1 start to 2.0 M, pH 5.0 final) at a flow rate of 1 ml/min (16). Five milliliter fractions were collected and the eluant between 90 and 105 ml was evaporated under nitrogen and dissolved in 0.2 M sodium citrate buffer, pH 2.2 for subsequent amino acid analysis and radioactivity determinations. The buffered sample was chromatographed on a Glenco Model 100 AS amino acid analyzer (Glenco Scientific, Houston, TX) equipped with a 0.2 x 60 cm column (DC4A resin, Durrum Chemical Corp., Palo Alto, CA). Elution was obtained

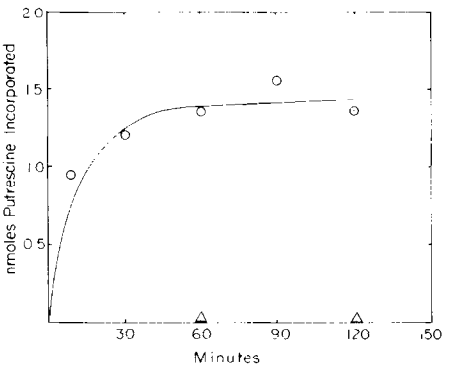


FIG. 1. Putrescine incorporation into guinea pig liver transglutaminase. Fifty micrograms of enzyme was incubated in the presence of 1 mM [$^{14}$C] putrescine (0.7 μCi/μmole), the presence (⊙) and absence (△) of $CaCl_2$ as described in Materials and Methods.

using the Pico II buffer system (Pierce Chemical Co., Rockford, IL) excluding the additive from buffer B. In this system the isopeptide elutes in the valley between methionine and isoleucine. The column effluent was monitored using Fluoropa (Durrum), a Schoeffel 970 fluorescence detector (Schoeffel Instrument Corp., Westwood, NJ) and an Autolab SP4000 computer-integrator system (Spectra Physics, Santa Clara, CA).

Putrescine incorporation into transglutaminase was measured in a 2 ml reaction mixture as described above except that 50 μg transglutaminase was used and 1 mM [1,4 $^{14}$C] putrescine (New England Nuclear, Boston, MA) was present. Transglutaminase activity was determined using casein as acceptor as described previously (11). Protein was estimated by the method of Lowry et al. (17). Specific activity is defined as units/mg protein with casein as acceptor.

## RESULTS AND DISCUSSION

Figure 1 illustrates the rate of 1 mM [$^{14}$C] putrescine incorporation into purified guinea pig liver transglutaminase. Incorporation reached a maximum after 60 min and was inhibited more than 95% when $Ca^{++}$ was omitted and 1 mM EDTA was added to the reaction mixture. Putrescine incorporation into casein was inhibited under similar conditions (data not shown).

The observation of putrescine incorporation into purified transglutaminase demonstrated the presence of amine acceptors in transglutaminase and

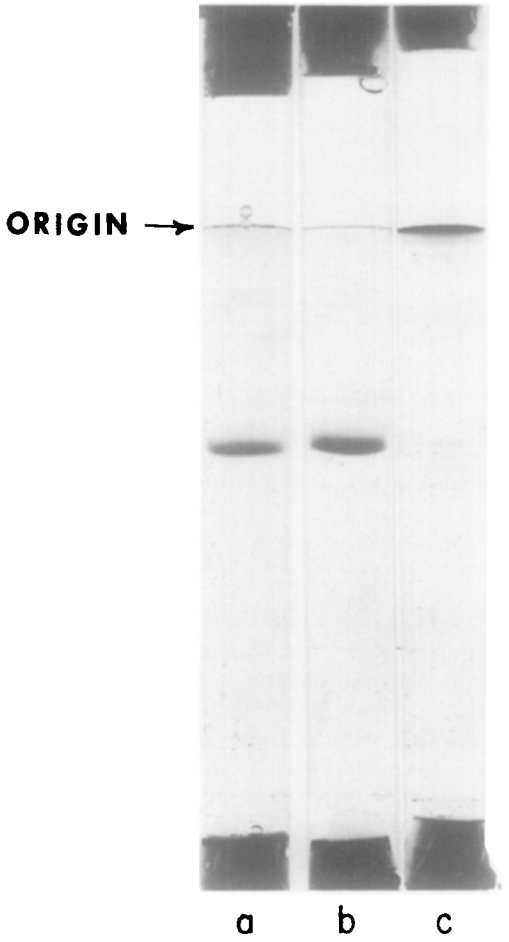


FIG. 2. Sodium dodecylsulfate polyacrylamide gel electrophoresis pattern of guinea pig liver transglutaminase. Each sample layered contained about 6 μg protein. Prior to electrophoresis samples were treated as described under Materials and Methods.
a) no $CaCl_2$, 1 mM EDTA, 37°, 60 min; b) no $CaCl_2$, 1 mM EDTA, 0 min; c) 5 mM $CaCl_2$, no EDTA, 37°, 60 min.

raised the possibility that the enzyme might catalyze isopeptide bond formation with itself. Figure 2 (gel b) shows that purified transglutaminase gave rise to a single band upon sodium dodecylsulfate polyacrylamide gel electrophoresis with an apparent molecular weight of 85-90,000 daltons in agreement with the findings of other workers (13). The gel pattern remained

essentially unchanged when the enzyme was incubated at 37° for 60 min in the presence of mM EDTA (gel a). Incubation of transglutaminase under catalytic conditions (5 mM $CaCl_2$, 37°, 60 min), however, resulted in the appearance of material with a larger apparent molecular weight (gel c). It is difficult to know how many species are present after calcium-catalyzed polymerization since molecules with a molecular weight greater than 250,000 will not enter the polyacrylamide gel. While a faint band was evident migrating just behind transglutaminase, the gels showed this material was not involved in the polymerization reaction.

Appearance of polymerized material in gel c suggested the formation of intermolecular crosslinks. Isopeptide formation was confirmed by the isolation of 33.1 and 28.2 nmoles ε-(γ-glutamyl)lysine/mg protein from proteolytic digests of two polymerized samples. In samples lacking $CaCl_2$, no ε-(γ-glutamyl)lysine was detected. If isopeptide was present, the level would have been less than 0.01 nmole/mg protein. Connellan et al. (13) previously reported that transglutaminase chromatographed with calcium at 4° showed no change in molecular weight. In our hands samples incubated at 4° showed no polymerization (data not shown).

From the amino acid composition of the purified enzyme (2, 13) and the isopeptide content of polymerized samples, about 8% of the lysine moieties are crosslinked to glutamine after 60 min. This value is equivalent to about 3 crosslinks per 90,000 daltons protein and would produce a polymer too large to enter the gel (gel c of Fig. 2).

The observation of putrescine incorporation into transglutaminase as well as isopeptide crosslink formation in the absence of putrescine raise some interesting biological possibilities for the enzyme: (a) Transglutaminase may act as an amine donor or amine acceptor substrate in a crosslink reac-

tion with itself or another protein molecule; such a reaction may be involved in control of enzyme activity since preliminary findings show polymerized transglutaminase has reduced enzyme activity. (b) Cellular polyamines may combine with the enzyme and regulate its activity. Cells reportedly having elevated polyamine levels possess lower transglutaminase activity than normal counter parts (11, 12). In conjunction with this, we are currently exploring the possibility of the existence of polyamines covalently bound to protein (e. g., γ-glutamylputrescine).

Finally, workers utilizing transglutaminase to crosslink proteins or label proteins with primary amines should be cognizant of these transglutaminase autocatalysis reactions in interpretations of their findings.

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