Hydrolysis of γε Isopeptides by Cytosolic Transglutaminases and by Coagulation Factor XIII⁎

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N⁎-(γ-glutamyl)lysine cross-links, connecting various peptide chain segments, are frequently the major products in transglutaminase-catalyzed reactions. We have now investigated the effectiveness of these enzymes for hydrolyzing the γε linkage. Branched compounds were synthesized, in which the backbone on the γ-side of the cross-bridge was labeled with a fluorophor (5-(dimethylamino)-1-naphthalenesulfonyl or 2-aminobenzoyl) attached through an ε-aminocaproyl linker in the N-terminal position, and the other branch of the bridge was constructed with Lys methylamide or diaminopentane blocked by 2,4-dinitrophenyl at the N⁎ position. Hydrolysis of the cross-link could be followed in these internally quenched substrates by an increase in fluorescence. In addition to the thrombin and Ca2+–activated human coagulation Factor XIIIγ, cytosolic transglutaminases from human red cells and from guinea pig liver were tested. All three enzymes were found to display good isopeptidase activities, with Km values of 10⁻⁴ to 10⁻⁵ M.

Inhibitors of transamidation were effective in blocking the hydrolysis by the enzymes, indicating that expression of isopeptidase activity did not require unusual protein conformations. We suggest that transglutaminases may play a dynamic role in biology not only by promoting the formation but also the breaking of N⁎-(γ-glutamyl)lysine isopeptides.

Apart from obvious differences in substrate specificities for the α-carbonyl groups of endo-Lys and Arg residues by papain (EC 3.4.22.2) and for the γ-carbonyl groups of certain endo-Gln residues by transglutaminases (EC 2.3.2.13), considerable kinetic and mechanistic similarities exist between these two families of enzymes. Both operate by acylation-deacylation pathways, with a Cys thiol in the catalytic center assisted by a His residue (1–6). However, because of the exceptional specificities of transglutaminases for amines mimicking the ε-amino groups of Lys side chains in proteins (7–9), this group of enzymes shows a unique ability for generating protein-to-protein N⁎-(γ-glutamyl)lysine cross-links, a post-translational reaction of major biological significance. Transglutaminases are known to participate in various clotting phenomena (7, 10–16), in the assembly of extracellular matrices (17) and of intracellular polymeric structures in cells under Ca2+ stress (18–22), and in apoptosis (22).

While a great deal of attention has been paid to the amine transferase activities of transglutaminases (3, 4, 24), i.e. to the production of N⁎-(γ-glutamyl)lysine bridges and the incorporation of small molecular weight amines into proteins, the isopeptide breaking potential of the enzymes has not yet been explored. Since lack of availability of appropriate substrates may have been a main reason, we embarked on synthesizing γ-branched peptides with built-in features, which would facilitate the application of fluorescence methodologies for kinetic studies. Two cytosolic transglutaminases of different properties, isolated from human red blood cells (HTg)1 and from guinea pig liver (GTg), respectively, and a recombinant form of the human coagulation Factor XIIIγ (rAγ⁎) were employed as enzymes.

MATERIALS AND METHODS

Organic Synthesis

Reagents, solvents, and blocked amino acid derivatives were purchased from Aldrich, Sigma, and Bachem Bioscience Inc. TLC (thin-layer chromatography) was performed on Whatman KFs-Silica gel glass plates (0.25 mm) using the following solvent systems (v/v): (A) chloroform/methanol/glacial acetic acid (10:3:1); (B) chloroform/methanol/2-propanol (10:4:4); (C) n-butanol/glacial acetic acid/water (15:5:5); (D) 1-propanol/water (7:3); (E) propanol/water/concentrated ammonium hydroxide/ethanol (7:4:2:3). Plates were viewed under UV light (at 254 nm and 366 nm for detection of UV absorbing and fluorescent moieties, respectively) or were developed by ninhydrin (0.25% in 1-butanol for N-deblocked peptides) or by hypochlorite (10%) followed by starch/KI spray for N-blocked peptides (26). Melting points were determined with a Büchi apparatus and are uncorrected. After acid hydrolysis, amino acid analyses were kindly carried out by Dr. Thomas J. Lukas of the Department of Molecular Pharmacology and Biochemistry, Northwestern University Medical School, Chicago, IL. Elemental analyses were performed by G. D. Searle Laboratories, Skokie, IL.

Peptide Coupling

To a stirred and cooled (0 °C) 0.5–0.8 M solution of the pertinent Boc amino acid in dry DMF were added equimolar amounts of 1-hydroxybenzotriazole and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. The mixture was stirred at 0 °C for 40 min and then added to a solution of the trifluoroacetate salt of the peptide benzyl ester

1 The abbreviations used are: HTg, human red blood cell transglutaminase; GTg, guinea pig liver transglutaminase; Boc, tert-butyloxycarbonyl; Z, benzoyloxycarbonyl; Dns or dansyl, 5-(dimethylaminomethyl)-1-naphthalenesulfonyl; Cad, cadaverine (or 1,5-diaminopentane); Dns-Cad or Cad-Dns, dansylcadaverine (or N-(5-diaminovaleryl)-5-(dimethylamino)-1-naphthalenesulfonylamide); Eaa, ε-aminocaproyl; O-Bzl, benzyl ester; Dnp, 2,4-dinitrophenyl; Dnp-Cad or Cad-Dnp, di-nitrophenylcadaverine; Abz, 2-aminobenzoyl; DNF, N,N-dimethylformamide; pGlu, pyroglutamyl; Dde or dabcyl, 4-(4'-dimethylaminophenylazo)benzoyl; rAγ, the recombinant human Factor XIII subunit A dimer; DTT, dithiothreitol; McCN, acetonitrile; HPLC, high performance liquid chromatography; GTP•S, guanosine 5’-O-(thio)triphosphate; dec., with decomposition.

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Deblocking of the Boc Group
To 1.0 mmol of the Boc-peptide benzyl ester was added 2 ml of 50% trifluoroacetic acid in anhydrous dichloromethane. The solution was allowed to stand at room temperature for 1 h, and excess trifluoroacetic acid was removed by adding fresh dichloromethane to the mixture followed by evaporation under reduced pressure, then by addition of anhydrous ether to the residue. The precipitated trifluoroacetic acid salt of the peptide benzyl ester was filtered off, washed with anhydrous ether, and the solvent evaporated to give the product.

Deblocking of the Benzyl Ester Group
The blocked peptide benzyl ester was dissolved in a mixture of DMF/ethanol/water in the approximate volume ratio of 10:20:2 at 50 °C and hydrogenated in presence of 10% Pd/C catalyst for 3.5 h at 50 °C with stirring. After cooling to room temperature, water and 1 x NH4OH to pH 9 were added and the mixture filtered to remove the catalyst through a Celite filter pad. The filtrate was evaporated under reduced pressure, the residue was taken up in a minimum volume of absolute ethanol, and about 10 volumes of anhydrous ether added and cooled to 0 °C. The precipitated product was filtered off, washed with anhydrous ether, and dried under vacuum in a desiccator for 2 h before proceeding with the coupling reaction.

Compounds Used as First Substrates
Dns-Eaca-Gln-Glu-Ile-Val (I)—This was synthesized as described in Ref. 27.
Dns-Eaca-Glu-[γ-(Cad-Dnp)]-Gln-Ile-Val (II)—This was synthesized by a multistep procedure as follows. First, Boc-Glu-α-benzyl ester was coupled to mono-Z-cadaverine (24), essentially according to the general procedure above and the product isolated from an ethyl acetate extract of the reaction mixture to give an 80% yield of Boc-Glu-[γ-(Cad-Z)]-α-benzyl ester, melting point (m.p.) 68–71 °C, Rf 0.91 (B), 0.92 (C), 0.87 (D), and 0.89 (E). Catalytic hydrogenation of this intermediate followed by reaction with N-benzyloxycarbonyllysine in presence of NaHCO3 according to the general procedures gave Boc-Glu-[γ-(Cad-Z)] in 75% yield, Rf 0.34 (D), 0.80 (C), 0.66 (D), and 0.72 (E). This was coupled by the above general procedure with the trifluoroacetate salt of Gln-Ile-Val-O-Bzl (28) to give an 83% yield of Boc-Glu-[γ-(Cad-Z)]-Gln-Ile-Val-O-Bzl, m.p. 229–231 °C, Rf 0.91 (A), 0.89 (C), 0.82 (D), and 0.89 (E). This material was deblocked to remove the Boc group and coupled with equimolar amount of Dns-Eaca according to the general procedures to give the fluorescent peptide intermediate, Dns-Eaca-Glu-[γ-(Cad-Z)]-Gln-Ile-Val-O-Bzl, yield 51%, m.p. (dec.) 235–240 °C, Rf 0.94 (A), 0.88 (C), 0.86 (D), and 0.91 (E). This was catalytically hydrogenated to give Dns-Eaca-Glu-[γ-(Cad)]-Gln-Ile-Val-O-Bzl, Rf 0.26 (A), 0.31 (C), 0.55 (D), and 0.63 (E). This was then reacted in 50% aqueous ethanol with a 40% molar excess of 2,4-dinitrofluorobenzene and NaHCO3. The reaction mixture was stirred at 0 °C for 0.5 h and at room temperature for 14 h while protected from light. About 15 drops of concentrated Na2CO3 solution were added to the mixture to raise the pH to 9–9.5 (for decomposition). The precipitated product was filtered off, or separated by centrifugation, washed with 5% NaHCO3, water, and the solvent evaporated to give a 40% yield of the titled quenched fluorescent peptide, Dns-Eaca-Glu-[γ-(Cad-Dnp)]-Gln-Ile-Val, as a yellow solid, m.p. (dec.) 206–208 °C, Rf 0.93 (B), 0.81 (C), 0.82 (D), and 0.89 (E). Hydrogenation of this material gave Dns-Eaca-Glu-[γ-(Cad-Dnp)]-Gln-Ile-Val-O-Bzl (see Ref. 28) to give a 60% yield of Boc-Glu-[γ-(Cad-Dnp)]-Gln-Ile-Val-O-Bzl, yield 51%, m.p. (dec.) 235–240 °C, Rf 0.93 (B), 0.81 (C), 0.82 (D), and 0.89 (E). This was catalytically hydrogenated and reacted with 2,4-dinitrofluorobenzene by a similar procedure as above to give the titled fluorescent peptide (III), yield 67%, yellow solid, m.p. (dec.) 218–221 °C, Rf 0.15 (B), 0.73 (C), 0.68 (D), and 0.84 (E). Amino acid analysis gave Glu 2.37 (2), Ile 0.86 (1), and Val 0.76 (1).
Boc-Glu-[γ-(Cad-Dnp)]-Gln-Ile-Val (V)—Coupling of dansylcadaverine (Cad-Dna) with Boc-Glu-O-Bzl followed by hydrogenation according to the published procedure (30) gave Boc-Glu-[γ-(Cad-Dnp)]-Gln-Ile-Val, yellow solid, m.p. (dec.) 225–232 °C, Rf 0.64 (C), 0.56 (D), and 0.74 (E). Amino acid analysis gave Glu 2.17 (2), Ile 0.86 (1), and Val 0.76 (1).
Boc-Glu-[γ-(Cad-Dnp)]-Gln-Ile-Val (VII)—Coupling of dansylcadaverine (Cad-Dna) with Boc-Glu-O-Bzl followed by hydrogenation according to the published procedure (30) gave Boc-Glu-[γ-(Cad-Dnp)]-Gln-Ile-Val, yellow solid, m.p. (dec.) 225–232 °C, Rf 0.64 (C), 0.56 (D), and 0.74 (E). Amino acid analysis gave Glu 2.17 (2), Ile 0.86 (1), and Val 0.76 (1).
Pgl-Ala-Glu-[γ-(Cad-Dns)]-Gln-Ile-Val (VI)—The above peptide intermediate was deblocked to remove the Boc group and coupled with pGlu-Ala according to the general procedures to give pGlu-Ala-Glu-[γ-(Cad-Dns)]-Gln-Ile-Val-O-Bzl, yield 81%, m.p. (dec.) 276–278 °C, Rf 0.94 (A), 0.94 (B), 0.91 (C), 0.86 (D), and 0.91 (E). Catalytic hydrogenation of this material gave the titled fluorescent peptide, V, yield 84%, m.p. (dec.) 212–214 °C, Rf 0.8 (A), 0.8 (B), 0.84 (C), 0.74 (D), and 0.88 (E). Amino acid analysis gave Glu 2.1 (2), Ile 0.88 (1), and Val 0.88 (1).
Boc-Glu-[γ-(Cad-Dnp)]-Gln-Ile-Val (VIII)—First, Boc-Glu-[γ-(Cad-Dnp)]-Gln-Ile-Val (VII) was dissolved in a mixture of DMF/ethanol/water in the approximate volume ratio of 10:20:2 at 50 °C and hydrogenated in presence of 10% Pd/C catalyst for 3.5 h at 50 °C with stirring. After cooling to room temperature, water and 1 x NH4OH to pH 9 were added and the mixture filtered to remove the catalyst through a Celite filter pad. The filtrate was evaporated under reduced pressure, the residue was taken up in a minimum volume of absolute ethanol, and about 10 volumes of anhydrous ether added and cooled to 0 °C. The precipitated product was filtered off, washed with anhydrous ether, and dried in vacuo.
**Enzymatic Studies**

Guinea pig liver transglutaminase (GTg; Refs. 33 and 34) and human red cell transglutaminase (HTg; Refs. 35 and 36) were purified as described previously and stored at ~80°C. Protein concentrations for GTg were measured by absorbance at 280 nm (ε=15,800). Protein concentrations for HTg were determined with the BCA protein assay (Pierce), utilizing biocinchoninic acid with bovine serum albumin for standard. Calculations were based on molecular weights of 76,600 for GTg (37) and 80,000 for HTg (35).

**Transglutaminase activity** was measured in a CytoFluor model 2300 (Millipore, Bedford, MA), upgraded to a model 2350, by monitoring the rate of increase in fluorescence during the transglutaminase-catalyzed incorporation of dansylcadaverine (38) into N,N-dimethylsarcosine (excitation filter = 360 ± 40 nm; emission filter = 490 ± 40; sensitivity 6). Incubations were carried out at 37°C in a Millipore 96-well low fluorescence CytoPlate in 125-µl reaction mixtures which comprised 50 mM Tris-HCl, pH 7.5, 0.1 mM dansylcadaverine, 2 mg/ml N,N-dimethylsarcosine (Me,SO), 0.1 M CaCl2, and 0.0–0.124 mM GTg or HTg.

Recombinant human factor XIII A subunit (rA2; Ref. 39), a gift from Dr. Paul D. Bishop (Zymogenetics, Seattle, WA), was converted to rA2 by incubating 4–8 μM rA2 with 32–64 NIH units/ml human α-thrombin (a gift from Dr. J. W. Fenton III, New York State Department of Health, Albany, NY) in 50 mM N-methylmorpholine, pH 7.5, for 30 min at room temperature. Thrombin activity was then quenched by the addition of a 4-fold excess of hirudin (128–256 units/ml; Sigma). Protein concentration for rA2 was determined using absorbance at 280 nm (ε=14.9).

**Thin Layer and High Performance Liquid Chromatography (TLC and HPLC)**

Mixtures of 100 µl comprising 50 mM N-methylmorpholine-HCl, pH 7.5, ionic strength 0.1 (adjusted with NaCl), 100 µM Boc-Glu-γ-(cad-Dnp)-Gln-Ile-Val-Gly-Pro-Leu (VIII) or a-Dns-Lys-NHCH3 (XIII), 0.65 µM GTg, 1 mM DTT, and either 1 mM CaCl2 or 1 mM EDTA were incubated at 37°C for 120 min, when 2 µl of 100 mM EDTA were added to stop the reaction; 25-µl samples were spotted (2 x 10 mm) on a Polygram TLC plate (0.1-mm Polyamide-6, 20 x 20 cm; Macherey & Nagel, Alltech Associates, Deerfield, IL), and separation was accomplished in an ascending mode in aqueous 1% pyridine, pH 5.4, for 60 min (40). The dried plate was photographed under UV light (366 nm).

HPLC separations were also performed on the same mixtures. Approximately 50 µl of sample was mixed with 60 µl of 0.6 M perchloric acid and centrifuged (2 min, 15,600 x g), and 100 µl was injected onto an Ultrasphere C 8 column (Beckman, Fullerton, CA) using gradients formed with H2O (containing 0.1% trifluoroacetic acid) and MeCN (containing 0.1% trifluoroacetic acid); from injection to 20 min, linear increase of MeCN to 20%; 20 to 22 min, isocratic 30% MeCN; 22 to 32 min, linear increase of MeCN to 30%; at 32 min, MeCN was eliminated (0.1 min) and the column was then re-equilibrated with 0.1% trifluoroacetic acid in H2O for 15 min. Peaks were detected by absorbance at 220 nm and by fluorescence (λexc = 338 nm; λem = 560 nm), recorded on a Hewlett-Packard 3390A integrator and collected with a Poor detection fraction collector (ISCO, Lincoln, NE) set for detection of slope. Collected fractions were concentrated on a Savant (Farmingdale, NY) Speed-Vac concentrator.
Isopeptidase Activities of Transglutaminases

FIG. 1. GTg-catalyzed hydrolysis of the γε isopeptide bond in the branched substrate: Boc-Glu-[γε-(α-Dns-Lys-NHCH$_3$)]-Gln-Ile-Val-Gly-Pro-Leu. The substrate (marked as S, i.e. compound VIII, 100 μM) was incubated (37°C) with GTg (0.65 μM) in 50 mM N-methylmorpholine-NCl (pH 7.5, μ = 0.1 adjusted with NaCl) and 1 mM DTT either in the presence of 1 mM CaCl$_2$ (lane 2 in panel A and graph 2 in panel B) or of 1 mM EDTA (lane 1 and graph 1). After a reaction time of 2 h, separation of product (marked as P$_1$) was accomplished by Polyamide TLC (viewed under UV, panel A) and by HPLC on a C$_8$ column (monitored by dansyl fluorescence, panel B), as described under “Materials and Methods.” Identity of the fluorescent P$_1$ product as α-Dns-Lys-NHCH$_3$ was established in relation to compound XIII as reference (lane 3 and graph 3). Both P$_1$ and XIII exhibited the same $R_P$ ~ 0.6 on polyamide (origin of application marked as O), and eluted at the same position from the C$_8$ column (~14.5 min). Comparison of the substrate peaks (S) in graphs 1 and 2 showed that, under the conditions of this experiment, the hydrolytic reaction progressed to ~60% of completion.

FIG. 2. Quenching of fluorescence in the reaction of Dns-Eaca-Gln-Gln-Ile-Val with α-Dnp-Lys-NHCH$_3$ catalyzed by GTg. Dns-Eaca-Gln-Gln-Ile-Val (compound I, 0.2 mM) and Dnp-Lys-NHCH$_3$ (compound XII, 0.5 mM) were incubated at 37°C with 0.05 μM ( ), 0.10 μM (△), 0.21 μM (◇), or no (○) GTg in 1 mM CaCl$_2$, 1 mM DTT, and 50 mM Tris-HCl, pH 7.5. Fluorescence, measured on the CytoFluor (sensitivity 8), is expressed as a percentage of the initial fluorescence reading in the absence of GTg.

Isopeptide Formation Monitored by the Quenching of Fluorescence—Acceptor substrates were synthesized with a fluorescent N-terminal blocking group (Dns), whereas the donor substrates (cadaverine or Lys-NHCH$_3$) contained some quenching moiety (Dnp or Dbc) in the N-terminal position. Affinities of the new substrates for the enzymes were sufficiently favorable so that they could be employed at low enough concentrations for minimizing bimolecular quenching in the starting mixtures. However, as the coupling reaction for forming the γε product progressed with the addition of enzymes, a significant drop in fluorescence ensued. This was attributed to the intramolecular quenching effect exerted by the Dnp or Dbc group on the Dns fluorophore concomitant with forming the isopeptide linkage. This approach, tested for a variety of substrate pairs, proved to be highly sensitive for measuring the rate of isopeptide formation. Catalysis by the human red cell and the guinea pig liver enzyme was explored either with Dnp-cadaverine (XI), Dbc-cadaverine (XIV), or α-Dnp-Lys-NHCH$_3$ (XII) as donor, in conjunction with Dns-Eaca-Gln-Gln-Ile-Val (I) as the acceptor substrate. Figs. 2 and 3 illustrate the findings for the tissue type of transglutaminases. Fig. 2 pertains to the reaction of the Dns-labeled acceptor (I) with Dnp-Lys-NHCH$_3$ (XII) as promoted by guinea pig liver transglutaminase, whereas Fig. 3 presents the data for the human red cell enzyme-catalyzed reaction between I and Dbc-cadaverine (XIV). Dns-Eaca-Pro-Ala-Gln-Gln-Ile-Val (X) could be used as first substrate either with Dbc-cadaverine (XIV) or with Dnp-cadaverine (XI) as second substrate for following the coupling reactions catalyzed by rA$_2$ (data not shown). These results guided our synthetic work for designing γ-branched peptides for examining the isopeptide breaking potentials of transglutaminases.

Transglutaminase-catalyzed Breaking of the Isopeptide Bond—We synthesized a number of intramolecularly quenched compounds containing an isopeptide linkage (described under “Materials and Methods”) and followed the actions of the enzymes by the increase in fluorescence resulting from the release of the quenching moiety (Dnp) attached to the leaving amine group. On the γ-side of the backbone the substrates were either a Dns or an Abz fluorophor in an N-terminal position.

Figs. 4 and 5 show the progression curves for the hydrolysis of the Dns-Eaca-Glu-[γε-α-Dnp-Lys-NHCH$_3$]-Gln-Ile-Val (II) (10$^{-4}$ M) substrate with the guinea pig liver and of compound II with the human red blood cell enzyme, respectively. Approximately twice as much GTg (0.43 μM) was used for demonstra-
FIG. 3. Decrease of fluorescence in the HTg-catalyzed reaction between Dns-Eaca-Gln-Gln-Ile-Val and dabcylcadaverine. Dns-Eaca-Gln-Gln-Ile-Val (compound I, 0.1 mM) and dabcylcadaverine (Dbc-Cad, compound XIV, 25–100 µM) were incubated at 37 °C with HTg (0.55 µM) in 1 mM CaCl₂, 1 mM DTT, and 50 mM methylmorpholine-HCl, pH 7.5. Fluorescence, measured on the CytoFluor (sensitivity 7), is expressed as a percentage of the initial fluorescence reading in the absence of HTg.

FIG. 4. Increase of fluorescence accompanying the reaction of Dns-Eaca-Glu-[γ-(Cad-Dnp)]-Gln-Ile-Val with GTg. Dns-Eaca-Glu-[γ-(Cad-Dnp)]-Gln-Ile-Val (compound III, 100 µM) was incubated at 37 °C with GTg (0.43 µM) in 1 mM CaCl₂, 1 mM DTT, and 50 mM methylmorpholine-HCl, pH 7.5. Fluorescence, measured on the CytoFluor (sensitivity 8), is expressed as the increase in fluorescence compared with the initial fluorescence reading without enzyme.

FIG. 5. Reaction of HTg with Dns-Eaca-Glu-[γ-(Cad-Dnp)]-Gln-Ile-Val. HTg (0.55 µM) was incubated at 37 °C with 2 µM (○), 3 µM (■), 5 µM (▲), 8 µM (□), or 10 µM (□) Dns-Eaca-Glu-[γ-(Cad-Dnp)]-Gln-Ile-Val (compound II) in 1 mM CaCl₂, 1 mM DTT, 50 mM methylmorpholine-HCl, pH 7.5; for each experiment ionic strength was adjusted to 0.1 with NaCl. Fluorescence, measured on the CytoFluor (sensitivity 8), is expressed as the increase in fluorescence compared with the initial fluorescence reading in the absence of HTg.

FIG. 6. The rA₂*-catalyzed reaction of the Dns-Eaca-Ala-Glu-[γ-(Cad-Dnp)]-Gln-Ile-Val substrate. Dns-Eaca-Ala-Glu-[γ-(Cad-Dnp)]-Gln-Ile-Val (compound IX, 40 µM) was incubated at 37 °C with 0.5 µM (○), 1 µM (△), 2 µM (▲), or 4 µM (□) thrombin-treated rA₂ in 1 mM CaCl₂, 1 mM DTT, and 50 mM methylmorpholine-HCl, pH 7.0. Fluorescence, measured on the CytoFluor (sensitivity 8), is expressed as the increase in fluorescence compared with the initial fluorescence reading without rA₂ added.

The hydrolytic cleavage of compound III (0.1 mM) in Fig. 4 than the concentration of the enzyme (0.21 µM) for generating the highest rate of cross-bridge formation in the coupling reaction between compounds (I; 0.24 mM) and XII (0.5 mM), depicted by solid triangles (▲) in Fig. 2. Because of inherent ambiguities in comparing rate constants for the reaction of a single substrate (as in the experiment in Fig. 4) with those for a two-substrate reaction (as in Fig. 2), we did not attempt to draw kinetic comparisons between these different enzymatic processes.

Some of the γ-branched peptides also satisfied the more restrictive specificity requirement of human Factor XIIIa. Fig. 6 presents our data by for the hydrolysis of Dns-Eaca-Ala-Glu-[γ-(Cad-Dnp)]-Gln-Ile-Val (IX) by rA₂*, and Fig. 7 for the reaction of the same enzyme with Abz-Eaca-Glu-[γ-(Cad-Dnp)]-Gln-Ile-Val (IV). Because of the limitations of the CytoFluor plate reader, fluorescence of the Abz-blocked compound (λex = 320 nm and λem = 410 nm), could only be monitored in the spectrophotofluorimeter. Fig. 7 shows that the hydrolytic activity of rA₂* on the γ-branched peptide could be abolished by an active site-directed inhibitor of Factor XIIIa: 1,3,4,5-tetramethyl-2-[[(2-oxopropyl)thio]imidazolium chloride (41). At the same concentration (10⁻⁴ M), this compound also inhibited totally the hydrolysis of Dns-Eaca-Glu-[γ-(Cad-Dnp)]-Gln-Ile-Val (II) by the human red cell transglutaminase (5.5 × 10⁻⁷ M) measured in the CytoFluor under similar conditions (data not shown).

Hydrolysis of Dns-Eaca-Glu-[γ-(Cad-Dnp)]-Gln-Ile-Val (II) by the human red cell transglutaminase proceeded fastest in the pH 6.5–7.5 range (ionic strength = 0.1), with reaction rates falling off on either side of this pH range (Fig. 8). For the guinea pig liver enzyme, the apparent optimum for the hydrolysis of the same substrate was in the pH 5.5–7.5 range (data not shown).

Tissue transglutaminases are negatively regulated by GTP for incorporating amines into protein substrates (42–44). We tested the effects of equimolar GTP-Mg²⁺ complexes on the isopeptidase activities of the enzymes. As illustrated in Fig. 9, GTP was found to exert a very strong inhibitory effect on the hydrolysis of Dns-Eaca-Glu-[γ-(Cad-Dnp)]-Gln-Ile-Val (II) by...
the human red cell enzyme (I_{50} \sim 5-10 \times 10^{-6} \text{ M GTP}). However, even the \sim 90\% inhibition caused by 2 \times 10^{-5} \text{ M GTP-Mg^{2+}} at low Ca^{2+} (10^{-3} \text{ M}), could be overcome substantially at higher concentrations of Ca^{2+} (Fig. 10). This finding supports the concept (43) that the binding of GTP causes a reduction in the Ca^{2+} sensitivity of transglutaminase. The inhibitory effect of GTPyS was indistinguishable from that of GTP (data not shown).

Transglutaminases are known to catalyze the hydrolysis of protein or peptide-bound Gln to Ghu residues (45), and also to hydrolyze nitrophophyl and thiocholine esters (3, 4, 24). The hydrolytic nature of transglutaminases is brought even more to the forefront by the examples provided in this paper for the breaking of isopeptide linkages, with high affinities for the \gamma-branching substrates (e.g. \overline{K_{m}} \sim 10^{-5} \text{ M} for substrate II by the human red cell enzyme; Fig. 5). Inhibition by the active-site directed blocking agent: 1,3,4,5-tetramethyl-2-(2-oxopropyl)thioimidazolium chloride (Fig. 7) and also by GTP or GTPyS (Figs. 9 and 10), which modulate the Ca^{2+} sensitivities of cytosolic transglutaminases, show that the same functional domains are involved in the expression of isopeptidase activities as in the well studied transamidating reactions promoted by these enzymes.

Claims have been made (46, 47) and refuted (48) for having isolated an isopeptidase, called destabilase, from leech saliva with specificity for hydrolyzing the \gamma-(\gamma-glutamyl)lysine bonds between the \gamma-chains of solubilized fibrin. The primary structure of destabilase, derived from the cDNA clone, does not share significant homology with transglutaminase (49). Based on the findings described in the present paper, it may be suggested that if a select group of enzymes exists with properties that would uniquely define them as isopeptidases, they would probably also display transamidating activities, the characteristic attributes of transglutaminase. One of the enzymes, Factor XIIIa, which was shown in our experiments to...
exhibit isopeptidase activity (Figs. 6 and 7), has been reported to actually hydrolyze the cross-link formed between $\alpha_2$-plasmin inhibitor and fibrinogen, and to a lesser extent also the cross-link between the inhibitor and fibrin (50, 51). Altogether, our findings with the cytosolic enzymes suggest that transglutaminases may play a more dynamic role in cell biology than hitherto envisaged, not only by catalyzing the formation but also the breaking of $N^\epsilon$-(\gamma-glutamyl)lysine bonds.

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