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Specificity of transglutaminase-catalyzed peptide synthesis

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ABSTRACT

Biocatalytic methods for peptide synthesis are of high value due to the rapidly increasing approval of peptide-based therapeutics and the need to develop new analogs. Guinea pig liver transglutaminase (gTG2) catalyzes the cross-linking of peptides and proteins *via* the formation of γ-glutamyl-ϵ-lysyl isopeptide bonds. In this study, we investigate gTG2-catalyzed peptide bond formation between various amino acid-derived donor and acceptor substrates. Using LC–MS analysis, we demonstrate that gTG2 forms Gly-Xaa and D-Ala-Gly dipeptide products, confirming that its natural transamidation activity can be co-opted for peptide synthesis. An aromatic ester of Gly was the most efficient acyl-donor substrate tested; aromatic esters of D-Ala and L-Ala showed 50-fold lower reactivity or no reactivity, respectively. A computational strategy combining computational protein design algorithms and molecular dynamics simulations was developed to model the binding modes of donor substrates in the gTG2 active site. We show that the inability of gTG2 to efficiently catalyze peptide synthesis from donors containing alanine results from the narrow substrate binding tunnel, which prevents bulkier donors from adopting a catalytically productive binding mode. Our observations pave the way to future protein engineering efforts to expand the substrate scope of gTG2 in peptide synthesis, which may lead to useful biocatalysts for the synthesis of desirable bioactive molecules.

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1. Introduction

The amide bond is among the most versatile functional groups in synthetic organic chemistry due to its high polarity, stability, and well-characterized conformational preference [1]. In particular, facile peptide bond formation – whether between natural or unnatural amino acids – is of extremely high value due to the rapidly increasing approval of peptide-based therapeutics and the need to develop new analogs. Conventional chemical approaches to peptide bond synthesis require chemical activation, protection, and deprotection steps for each bond formed as well as orthogonal protection of reactive substituents. As a result, peptide bond synthesis remains an important challenge in chemistry [2]. Enzymatic approaches have attempted to alleviate these limitations. This is generally performed by running proteases "backward", toward bond synthesis rather than hydrolysis (recently reviewed in [3]). Despite engineering of proteases and optimization of reaction conditions, hydrolysis of existing peptide bonds reduces yield. Using an enzyme that has evolved to synthesize an amide bond, rather than hydrolyze it, could prove advantageous in enzyme-catalyzed peptide bond synthesis.

One such enzyme is tissue transglutaminase (TG2), which catalyzes the Ca^{2+}-dependent cross-linking of peptides and proteins *via* the formation of γ-glutamyl-ϵ-lysyl isopeptide bonds [4–6]. The catalytic reaction follows a modified ping-pong mechanism in which a glutamine-containing protein or peptide, the acyl-donor substrate, reacts with the catalytic cysteine residue to form a

Abbreviations: 7HC, 7-hydroxycoumarin; Cbz, carbobenzyloxy; TG2, tissue transglutaminase; MD, molecular dynamics; GABA, γ-aminobutyric acid; gTG2, guinea pig liver transglutaminase; NTA, nitrilotriacetic acid; MOPS, 3-(*N*-morpholino) propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DMF, *N*,*N*-dimethylformamide; HRMS, high-resolution mass spectrometry; ESI, electrospray ionization; FAB, fast atom bombardment; LC–MS, liquid chromatography-mass spectrometry; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; MOE, molecular operating environment; Boc, *tert*-butyloxycarbonyl.

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thioester bond. The resulting covalent acyl-enzyme intermediate then reacts with a second substrate, the acyl-acceptor, to yield the isopeptide-containing product and free enzyme in a transamidation reaction. In the absence of an amine acyl-acceptor, the acyl-enzyme intermediate can be hydrolyzed, transforming the acyl-donor glutamine residue into glutamate and regenerating the free enzyme [7].

TG2 enzymes exhibit broad specificity toward the acyl-acceptor substrate [8]. Although the native acyl-acceptor substrate is generally a lysine-containing protein or peptide, many non-natural primary amines, such as glycinamide [9,10], and anilines, such as *N*,*N*-dimethyl-1,4-phenylenediamine [11], can also react. However, amines containing free carboxylic acid groups, such as free amino acids, do not act as substrates [10]. On the other hand, TG2 displays narrow specificity for its acyl-donor substrates. The side chain of a protein or peptide-bound L-Gln residue is the native substrate while the side chain of the similar amino acid L-Asn is not reactive [9]. In addition to amides, γ-glutamyl aromatic ester derivatives of L-Glu, such as *N*-carbobenzyloxy-L-glutamyl(γ-*p*-nitrophenyl ester) glycine (Fig. 1A), have also been shown to be acyl-donor substrates of TG2 and are used to measure the enzyme's activity [12]. However, secondary amide derivatives of L-Gln, such as *N*-γ-methyl-L-glutamine or anilides, are not substrates of TG2 [13]: the γ-carboxamide group of L-Gln is the only known amide that is an acyl-donor substrate of TG2.

We and others previously demonstrated that TG2 could use a novel class of acyl-donor substrates that are neither L-Gln nor L-Glu derivatives [14,15]. Namely, 4-(*N*-carbobenzyloxyglycylamino)-butyric acid-coumarin-7-yl ester (Cbz-Gly-GABA-7HC) and 4-(*N*-carbobenzyloxyphenylalanylamino)-butyric acid-coumarin-7-yl ester (Cbz-Phe-GABA-7HC) (Fig. 1B) can react with TG2 to release 7-hydroxycoumarin (7HC), resulting in a fluorescence increase that makes these compounds useful for quantifying TG2 reaction rates. The scaffolds of these substrates, based on known irreversible inhibitors of TG2 [16,17], differ from L-Glu aromatic ester acyl-donor substrates of TG2 in that the reactive ester function is located on the main chain of the peptide analog, rather than on the side chain. As a result, they give rise to products that do not contain a γ-glutamyl-ϵ-lysyl isopeptide bond. An analog in which the 7HC leaving group is attached directly to the glycine residue carboxylate group, *N*-carbobenzyloxyglycyl-coumarin-7-yl ester (Cbz-Gly-7HC, Fig. 1C), is also a donor substrate of TG2 [18]. Significantly, the reaction of this substrate with an acceptor amine substrate would result in the formation of a peptide-like α-amide bond (Scheme 1). These results illustrate that specificity for acyl-donor substrates with aromatic ester functions is broader than had previously been supposed and demonstrate that the enzyme can generate products with novel scaffolds.

In this study, we investigate guinea pig liver TG2 (gTG2)-catalyzed peptide bond formation between the Cbz-Gly-7HC donor substrate in combination with various amino acid-derived acceptors. Using LC–MS analysis of the reaction products, we demonstrate that the enzyme is able to react directly with the α-carboxyl group of Cbz-Gly-7HC to form Gly-Xaa dipeptide products, confirming that its natural transamidation activity can be co-opted for peptide synthesis. Additionally, we explore the substrate specificity of the enzyme in peptide synthesis by measuring its reactivity toward a variety of potential acyl-donor substrates having an aromatic ester function on the α-carboxyl group of various amino acids. We observed that the aromatic ester of Gly is an efficient acyl-donor substrate; the aromatic ester of D-Ala is also reactive though to a lesser extent, and that of L-Ala showed no detectable reactivity.

To elucidate how the stereochemical configuration of the side-chain of alanine-containing donor substrates affects gTG2 catalytic efficiency, we used a computational strategy combining computational protein design and molecular dynamics simulations to model the binding modes of donors in the gTG2 active site. We show that the inability of gTG2 to efficiently catalyze peptide synthesis from donors other than Cbz-Gly-7HC results from the narrow substrate binding tunnel, which prevents bulkier donors to adopt a catalytically productive binding mode. Our observations pave the way to future protein engineering efforts to expand the substrate scope of gTG2 in peptide synthesis, which may lead to useful biocatalysts for the synthesis of desirable bioactive molecules.

2. Materials and methods

2.1. Materials

All reagents used were of the highest available purity. Lysozyme, 7HC, *N*-acetyl-L-lysine methyl ester hydrochloride (*N*-AcLysOMe), *N*-carbobenzyloxy-L-glutaminylglycine (Cbz-L-Gln-Gly), glycinamide ($GlyNH_2$) and L-leucine methyl ester (LeuOMe) hydrochlorides were purchased from Sigma–Aldrich. L-Alaninamide hydrochloride ($AlaNH_2$) was purchased from Novabiochem (Mississauga, ON). Ni-NTA agarose resin was purchased from Qiagen (Mississauga, ON). Restriction enzymes and DNA-modifying enzymes were from New England Biolabs. All aqueous solutions were prepared using water purified with a Millipore BioCell system.

2.2. Synthesis of donor substrates

2.2.1. Synthesis of Cbz-Gly-7HC and Cbz-L-Ala-7HC

The synthesis of Cbz-Gly-7HC was based on a previously reported protocol [14]. Namely, 0.2 g (1 mmol) of Cbz-Gly and 0.4 g (2.5 mmol) of 7HC were dissolved in 10 mL of ethyl acetate. Then, 0.22 mL (0.2 g, 2 mmol) of *N*-methylmorpholine and 0.8 mL (0.63 g, 5 mmol) of *N,N*-diisopropylcarbodiimide were added with stirring at room temperature. Stirring was continued until the complete disappearance of Cbz-Gly, as followed by thin layer chromatography (ethyl acetate). The reaction mixture was then washed once with 1 M NaOH, three times with 0.1 M NaOH, 3 times with 0.1 M HCl, once with saturated $NaHCO_3$, and once with brine. The organic phase was then dried over $MgSO_4$, filtered and evaporated under reduced pressure. The resulting residue was purified by silica gel chromatography (ethyl acetate) to remove traces of diisopropylurea, giving the desired ester in 70% yield (0.25 g). Cbz-L-Ala-7HC was synthesized according to a similar protocol.

Cbz-Gly-7HC. ^1H NMR (300 MHz, $CDCl_3$): δ 4.30 (2H, d), 5.18 (2H, s), 5.33 (1H, s), 6.44 (1H, d), 7.08 (1H, d), 7.10 (1H, s), 7.37 (5H, m), 7.51 (1H, d), 7.70 (1H, d). ^{13}C NMR (75 MHz, CDCl3): δ 168.5, 160.6, 156.7, 154.9, 152.9, 143.1, 136.3, 129.0, 128.9, 128.6, 128.5, 118.4, 117.2, 116.6, 110.5, 67.7, 43.2. HRMS (FAB) calculated for $C_{19}H_{16}NO_6$ ($[M^+H]^+$): 354.0972, found 354.0968.

Cbz-L-Ala-7HC. ^1H NMR (300 MHz, $CDCl_3$): δ 1.63 (3H, d), 4.62 (1H, m), 5.18 (2H, s), 5.33 (1H, d), 6.42 (1H, d), 7.07 (1H, d), 7.09 (1H, s), 7.39 (5H, m), 7.51 (1H, d), 7.70 (1H, d). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.5, 160.5, 156.0, 154.9, 153.1, 143.1, 136.4, 129.0, 128.9, 128.6, 128.5, 118.4, 117.2, 116.6, 110.5, 67.5, 50.2, 18.5. HRMS (FAB) calculated for $C_{20}H_{18}NO_6$ ($[M^+H]^+$): 368.1129, found 368.1118.

2.2.2. Synthesis of Cbz-D-Ala-7HC

The synthesis of Cbz-D-Ala-7HC followed the protocol employed for Cbz-L-Ala-7HC. Equimolar amounts of Cbz-D-Ala (4 mmol, 0.89 g) and 7HC (4 mmol, 0.65 g) were dissolved in 15 mL of dichloromethane at room temperature. To the stirring solution, 0.38 mL (4.4 mmol) of *N*-methylmorpholine and 0.82 mL (8 mmol) of *N,N*-diisopropylcarbodiimide were added. The consumption of Cbz-D-Ala was monitored by thin-layer chromatography. Upon completion, the reaction mixture was washed successively with

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