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Glutaminyl Cyclases Display Significant Catalytic Proficiency for Glutamyl Substrates

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ABSTRACT: N-Terminal glutaminyl and glutamyl residues of peptides and proteins tend to form pyroglutamic acid (pGlu) by intramolecular cylization. The rate constants for spontaneous cyclization of glutamine (10^{-6} s^{-1}) and glutamic acid (10^{-9} s^{-1}) in aqueous solution differ by ~3 orders of magnitude at pH 6.5. Glutaminyl cyclases (QCs) from plants and mammals accelerate pGlu formation. Human QC exhibits a rate enhancement of 2.2×10^5 for glutamate cyclization, approximately 2 orders of magnitude lower than that of the corresponding N-terminal glutaminyl reaction. Thus, glutaminyl cyclases are enzymes with only modest specificity for cyclization of their primary glutaminyl substrates and may provide a link between glutamate cyclization and pathophysiology.

Pyroglutamic acid (pGlu, 5-oxo-L-proline) is a cyclic nonproteinogenic amino acid, which is generated from glutamine or glutamic acid by deamidation or dehydration, respectively (*1*−*3*). It has been shown that these reactions can be enzymatically catalyzed by glutaminyl cyclases (QCs). Previous investigations of the substrate conversion by QCs indicate that N-terminal formation of pyroglutamate represents an irreversible reaction (*4*, *5*). Formation and accumulation of pGlu-modified amyloid peptides occur in Alzheimer's disease and familial British dementia, and QC inhibition suppresses pGlu-amyloid peptides in mouse models of AD (*6*). As a means of developing a complete understanding of pGlu-amyloid formation, it was our aim to assess the catalytic performance of QCs for both deamidation and dehydration reactions.

As a result of N-terminal glutaminyl and glutamyl conversion into pGlu, the N-termini of peptides lose their basicity. The former amino group is incorporated into the lactam ring, which does not serve as a proton acceptor (Scheme 1). We analyzed conversion of N-glutaminyl and N-glutamyl peptides into pGlu products using capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC). Linear progress curves of QC-catalyzed conversion of Aβ(3−6) (sequence, EFRH) and Aβ(3−11) (sequence, EFRHDSGYE) were obtained, and the initial velocity of the QC-mediated turnover of Aβ(3−6), Aβ(3[Q]−6) (sequence, QFRH), and Aβ(3−11) was found to be in accordance with Michaelis−Menten kinetics (Figure 1 of the Supporting Information).

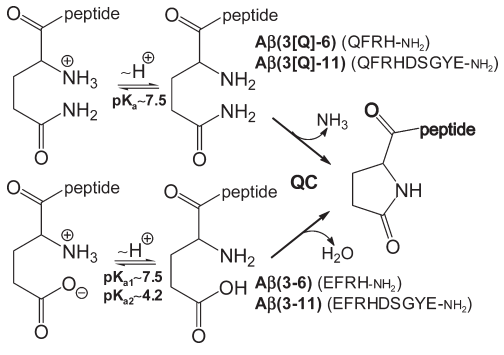
The kinetic parameters obtained for conversion of EFRH by human QC at pH 6.5 [i.e., $K_M = 6.0 \pm 2.4$ mM, and $k_{cat} = (2.2 \pm 0.5) \times 10^{-4}$ s^{-1}] were 10 times higher and 1.5×10^5-fold lower, respectively, compared to those of the Gln substrate. The conversion of Aβ(3−11) was more efficient [$K_M = 4.6 \pm 1.2$ mM, and $k_{cat} = (4.2 \pm 1.2) \times 10^{-4}$ s^{-1}] with a specificity constant of $(9.0 \pm 0.7) \times 10^{-2}$ M^{-1} s^{-1} (see also Table S1 of the Supporting Information). The difference in k_{cat} for conversion of peptides bearing N-terminal glutamine and glutamic acid enabled the determination of the (inhibitory) influence of the Glu substrates on the QC-catalyzed cyclization of the fluorogenic substrate Gln-AMC. The data validate binding of the peptides in the active site of QC, blocking glutaminyl peptide conversion (Figure 2 and Table S2 of the Supporting Information). Conversion of EFRH by different QCs was investigated under first-order rate conditions, i.e., at $[S] \ll K_M$, allowing a direct estimation of k_{cat}/K_M (Table S1). The results obtained from nonlinear regression of hQC-catalyzed conversion of EFRH [Aβ(3−6) (Table S1) and $(9.0 \pm 0.7) \times 10^{-2}$ M^{-1} s^{-1} for Aβ(3−11)] agreed with the direct determination of k_{cat}/K_M [Table S1 and $(7.3 \pm 0.8) \times 10^{-2}$ M^{-1} s^{-1} for Aβ(3−11)] at low substrate concentrations, verifying the assumptions mentioned above.

The catalytic performance of QC for cyclization of N-terminal glutaminyl and glutamyl residues was determined from the fate of EFRH and QFRH in forming Aβ(3[pE]−6) (pEFRH) spontaneously.

The kinetics of glutaminyl cyclization agreed with a first-order model (Figure 1A). On the basis of these results, glutamyl cyclization was evaluated according to first-order rate law conditions. The half-life was extrapolated from the initial slope assuming that the pGlu accumulation at infinity equals the initial precursor concentration. The rate constants of cyclization of N-terminal glutamine and glutamic acid differed by approximately $1.5-5 \times 10^3$ s^{-1}, depending on the pH conditions (Figure 1B).

The pH dependence of spontaneous glutaminyl cyclization is not dramatically affected by the pH value of the solution. A slight decrease in the extent of cyclization was observed below pH 7.0 in

Scheme 1: Pyroglutamate Formation at the N-Terminus of Peptides[a]



[a]The pK_a values correspond to the N-terminal residues and were obtained from refs 4 and 7.

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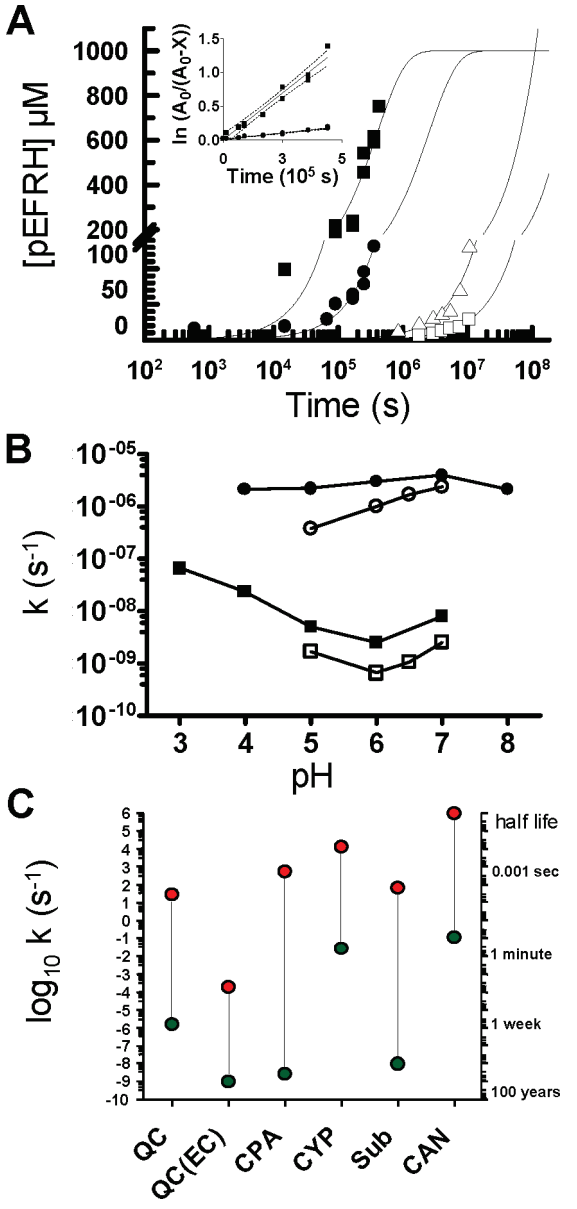

FIGURE 1: Spontaneous cyclization of N-terminal glutam(in)yl residues and rate enhancement by human QC at 30 °C. (A) pEFRH formation from glutaminyl (filled symbols; circles for pH 5.0 and squares for pH 7.0) and glutamyl residues (empty symbols; squares for pH 6.0 and triangles for pH 7.0). The inset shows the data for QFRH cyclization after linearization according to a first-order process. X denotes the concentration of substrate, which is converted into product at a certain time point. (B) Rate constants of spontaneous cyclization of EFRH (squares) and QFRH (circles). The cyclization was assessed in two different buffers, 0.1 M Mes with 0.5% Triton X-100 (filled symbols) and 0.05 M Mes, 0.1 M Tris, 0.05 M acetate, and 0.5% Triton X-100 (empty symbols) and HPLC (EFRH only) and CE (QFRH and EFRH) analysis to investigate the role of the buffer system and method of analysis. The rate constants were obtained by nonlinear regression according to a first-order process. (C) Rate constants for several spontaneous and enzyme-catalyzed reactions. The rate constants of noncatalyzed reactions are depicted in green and those of catalyzed reactions in red. Abbreviations: QC, human glutaminyl cyclase (glutaminyl cyclization); QC-(EC), human glutaminyl cyclase (glutamic acid cyclization); CPA, carboxypeptidase A; CYP, cyclophilin; Sub, subtilisin; Can, carbonic anhydrase. The data for QC were obtained from this work; the other rates are from refs 8 and 9.

Mes buffer [Figure 1B (○)]; however, the rate constants remained within 1 order of magnitude over the entire pH range investigated, supporting the reproducibility of the investigations under different buffer conditions. In contrast to glutaminyl cyclization, spontaneous formation of pGlu from N-terminal glutamic acid is significantly accelerated at low pH and is minimal at pH 6. The reason for the prominent pH dependence of spontaneous cyclization of glutamic acid has not been investigated in detail; however, it may reflect the requirement for both a protonated γ-carboxyl group and a deprotonated α-amino group for nucleophilic attack. Interestingly, the rate of spontaneous cyclization of glutamine and glutamic acid becomes similar at low pH values. Because the pK_a values of the amino group of glutamic acid and glutamine do not differ significantly, the concentrations of the protonated glutamine and glutamic acid residues are similar. Cyclization therefore may result from accelerated expulsion of water due to enhanced protonation of the leaving group, or from rapid formation of the tetrahedral intermediate secondary to protonation of the carboxyl side chain. Interestingly, the pH dependence of glutamyl cyclization is very similar to those observed recently for pGlu formation at the N-terminus of antibodies (*10*), revealing a minimal rate close to the pH optimum of the QC-catalyzed reaction at pH 6−7 (Figure S3 of the Supporting Information). Especially at pH's between the γ-carboxyl and α-amino pK_a values, the concentration of the deprotonated α-amino compound and the protonated γ-carboxylic residue is expected to be maximal. Such uncharged residues are likely candidates for binding of the QC active site as suggested by previous enzymatic pH dependence investigations (*11*).

Table 1: Catalytic Proficiencies of Different QCs at pH 6.5, Calculated Using First-Order Rate Constants (k_{non}) of $(1.7 \pm 0.2) \times 10^{-6}$ and $(1.0 \pm 0.4) \times 10^{-9}$ s^{-1} for Spontaneous Cyclization of Gln and Glu, Respectively

	peptide	k_{cat} (s^{-1})	k_{cat}/k_{non}	K_{tx}^{-1} (M^{-1})
human QC	QFRH	30 ± 1	$(1.7 \pm 0.2) \times 10^7$	$(5.9 \pm 0.5) \times 10^{10}$
	EFRH	$(2.2 \pm 0.5) \times 10^{-4}$	$(2.2 \pm 0.4) \times 10^5$	$(3.9 \pm 0.1) \times 10^7$
murine QC	QFRH	40 ± 1	$(2.3 \pm 0.2) \times 10^7$	$(7.9 \pm 0.9) \times 10^{10}$
	EFRH	nd[a]	nd[a]	$(1.9 \pm 0.1) \times 10^7$
potato QC	QFRH	56 ± 2	$(3.3 \pm 0.3) \times 10^7$	$(5.8 \pm 0.7) \times 10^{10}$
	EFRH	nd[a]	nd[a]	$(1.70 \pm 0.03) \times 10^8$
papaya QC	QFRH	42 ± 3	$(2.5 \pm 0.1) \times 10^7$	$(8.5 \pm 0.9) \times 10^{10}$
	EFRH	nd[a]	nd[a]	$(7.4 \pm 0.4) \times 10^8$

[a]Not determined.

On the basis of the noncatalyzed and enzyme-catalyzed conversion of N-terminal glutamine, QCs display a catalytic proficiency $(k_{cat}/K_M)/k_{non}$ (also denoted as K_{tx}^{-1}) of $5-9 \times 10^{10}$ M^{-1} and a rate enhancement of $\sim 2 \times 10^7$ relative to water. Thus, QCs exhibit a low proficiency compared with many hydrolases, decarboxylating enzymes or dehalogenating enzymes, but somewhat higher proficiencies than carbonic anhydrase or cyclophilin (Figure 1C) (*12*, *13*). Intriguingly, though, proficiency factors of $2-70 \times 10^7$ M^{-1} ($-\log K_{tx} = 7.3-8.9$) are still observed for catalysis of N-terminal cyclization of glutamic acid, because of the slower spontaneous cyclization of glutamic acid (Table 1).

Albeit 2−3 orders of magnitude below the proficiency of glutaminyl cyclization, the performance is on the order, for instance, of that of cyclophilin catalyzing the isomerization of prolyl peptide bonds ($-\log K_{tx} = 8.7$). Thus, even though the glutamyl cyclization might represent a nonphysiological reactivity, the catalysis appears to be relevant from an enzymological perspective. Interestingly, while plant glutaminyl cyclases catalyze the cyclization of glutamic acid with a proficiency significantly

higher than that of mammalian QCs, the structurally different QC enzyme groups exhibit similar cyclization of N-terminal glutamine (Table 1). These differing profiles may reflect alternate enzymatic mechanisms, a notion supported by the fact that insect and mammalian QCs act in a Zn^{2+}-dependent manner (*11*, *14*), in contrast to plant QCs which are metal-independent (*15*).

What are the implications of these findings for pyroglutamyl-peptide formation *in vivo*? From the current perspective, N-terminal cyclization of glutamic acid appears not to be important for the generation of higher concentrations of peptides, e.g., as required for hormonal action. Under certain pathophysiological conditions, however, the slow but catalyzed formation of pGlu from Glu precursors could manifest a disease relevant change for instance. Considering that the spontaneous conversion of glutamine and glutamic acid to pGlu at physiological pH requires on the order of 3−6 days and 9−34 years, respectively, a role for QCs is conceivable. With regard to the accumulation of pE-amyloid in Alzheimer's disease and familial British or Danish dementia, we would propose QC as a potential catalyst in glutamyl cyclization.

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SUPPORTING INFORMATION AVAILABLE

Detailed descriptions of the assay and generation of enzyme kinetic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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