Glutaminyl cyclases unfold glutamyl cyclase activity under mild acid conditions

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Abstract N-terminal pyroglutamate (pGlu) formation from glutaminyl precursors is a posttranslational event in the processing of bioactive neuropeptides such as thyrotropin-releasing hormone and neurotensin during their maturation in the secretory pathway. The reaction is facilitated by glutaminyl cyclase (QC), an enzyme highly abundant in mammalian brain. Here, we describe for the first time that human and papaya QC also catalyze N-terminal glutamate cyclization. Surprisingly, the enzymatic Glu^1 conversion is favored at pH 6.0 while Gln^1 conversion occurs with an optimum at pH 8.0. This unexpected finding might be of importance for deciphering the events leading to deposition of highly toxic pyroglutamyl peptides in amyloidotic diseases.

Key words: Amyloid precursor protein; Alzheimer's disease; Glutamine cyclotransferase; Glutaminyl cyclase; Glutamyl cyclase

1. Introduction

Regulatory peptides such as gonadotropin-releasing hormone (GnRH), thyrotropin-releasing hormone (TRH) and neurotensin, and the cytokines MCP-1–4, require N-terminal pyroglutamate in order to exert their respective biological functions [1,2]. Early studies have suggested that the formation of pyroglutamate at the N-terminus of Gln^1 peptides was a spontaneous reaction [3]. Glutaminyl cyclase (QC; EC 2.3.2.5), however, was identified to be the responsible physiological catalyst (Scheme 1) [4–7].

The first QC was isolated by Messer from the latex of the tropical plant *Carica papaya* in 1963 [4]. Later, in 1987, a corresponding enzymatic activity was discovered in animal pituitary. This mammalian QC was shown to convert the Gln^1 precursors of TRH and GnRH into the appropriate mature pyroglutamic acid ($pGlu^1$) peptides [5,7]. In addition, QC was co-localized to the secretory pathway of the bovine pituitary together with its putative products of catalysis, supporting its processing role in peptide hormone biosynthesis [8].

Coincidentally, in several neurodegenerative disorders pyroglutamate-containing peptides are thought to contribute to the pathogenesis by enhancing the proteolytic stability and neurotoxicity of hydrophobic, plaque-forming peptides [9]. The most prominent severe dementia, Alzheimer's disease (AD), is characterized by abnormal accumulation of extracellular amyloid plaques [10–12]. Amyloid-β (Aβ) peptides are generated by proteolytic processing of the β-amyloid precursor protein (APP), which is cleaved N-terminally by β-secretase and C-terminally by γ-secretase in a subsequent step [13].

Interestingly, within the core of amyloid plaques there exists a dominant fraction of Aβ peptides containing N-terminal pGlu, e.g. $pGlu^3$-Aβ(3–40/42) and $pGlu^{11}$-Aβ(11–40/42). These shortened peptides are reported to be more neurotoxic and to aggregate more rapidly than the full-length isoforms in vitro [9,14–16].

Moreover, among all prominent Aβ peptides, the isoforms containing pyroglutamate at position 3 represent the most abundant (~50% of total Aβ protein) of the N-terminally truncated peptide species [17–23]. This accumulation of pGlu-Aβ peptides is likely due to the structural modification that enhances aggregation and confers resistance to most aminopeptidases [15–20,24,25].

In order to prove whether QC is a candidate enzyme able to recognize and to turn over Aβ-derived peptides, we synthesized and investigated Gln^3-Aβ(1–11)a, Aβ(3–11)a, Gln^3-Aβ(3–11)a, Aβ(3–21)a, Gln^3-Aβ(3–21)a and Gln^3-Aβ(3–40) as potential substrates of the enzyme. Another objective of the study was to compare the fate of the Aβ-derived peptides using QC in combination with an aminopeptidase, under the rationale that aminopeptidase cleavage of full-length Aβ peptide is a prerequisite for QC cyclization of Glu/Gln at position 3 from the N-terminus. Finally, it was of interest whether such QC processing of the above Aβ peptides can be suppressed by recently characterized QC inhibitors [26].

2. Materials and methods

2.1. Enzymes

Human QC was expressed in *Pichia pastoris* or *Escherichia coli* and purified as described [27]. Papaya QC was purified from papaya latex essentially as described elsewhere [28]. Purified porcine dipeptidyl peptidase IV (DPIV) and pituitary homogenate were gifts from M. Wermann and L. Wagner (Probiodrug).

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Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; Aβ(3–11)a, amyloid-β peptide 3–11 amide; βNA, 2-naphthylamine; DPIV, dipeptidyl peptidase IV; EC, glutamyl cyclase; GnRH, gonadotropin-releasing hormone; pGlu, pyroglutamic acid; QC, glutaminyl cyclase; TRH, thyrotropin-releasing hormone

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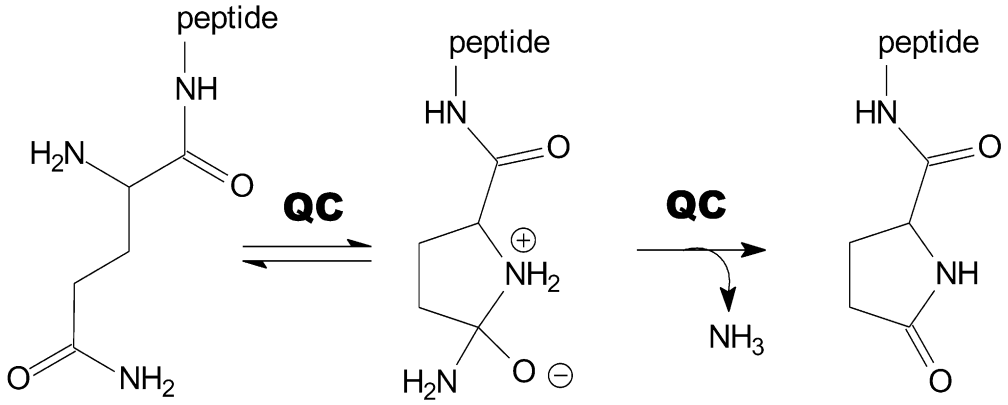


Scheme 1. N-terminal cyclization of glutaminyl peptides by QC.

2.2. Oligopeptide synthesis

Aβ peptide fragments Gln^3-Aβ(1–11)a, Aβ(3–11)a, Gln^3-Aβ(3–11)a, Aβ(3–21)a and Gln^3-Aβ(3–21)a were synthesized as C-terminal amides both semi-automatically in a 0.5 mmol scale on a peptide synthesizer (Labortec SP650, Bachem) as previously described [27] or using a Symphony peptide synthesizer (Rainin Instruments) in a 50 μmol scale on NovaSyn®TGR resin (0.23 mmol/g). Aβ peptide Gln^3-Aβ(3–40) was synthesized in 25 μmol scale on Fmoc-Val-NovaSyn®TGA resin (0.15 mmol/g). Preparative high performance liquid chromatography (HPLC) was performed with a gradient of acetonitrile in water (20–65% acetonitrile over 40 min) on a 250-20 Luna RP18 column (Phenomenex). Peptide purity and identity was confirmed by analytical HPLC and electrospray ionization mass spectrometry.

2.3. Assays of QC

QC activity was evaluated spectrophotometrically and fluorometrically at 30°C [27–29]. Glu-2-naphthylamine (βNA) was employed for observation of glutamate cyclization catalyzed by papaya QC. Spontaneous cyclization of Glu-βNA and Gln-βNA (2.0 mM) was investigated for 30 days in 20 mM MES buffer, pH 6.0, 30°C. Samples were removed for determination of pGlu content, diluted 10-fold and

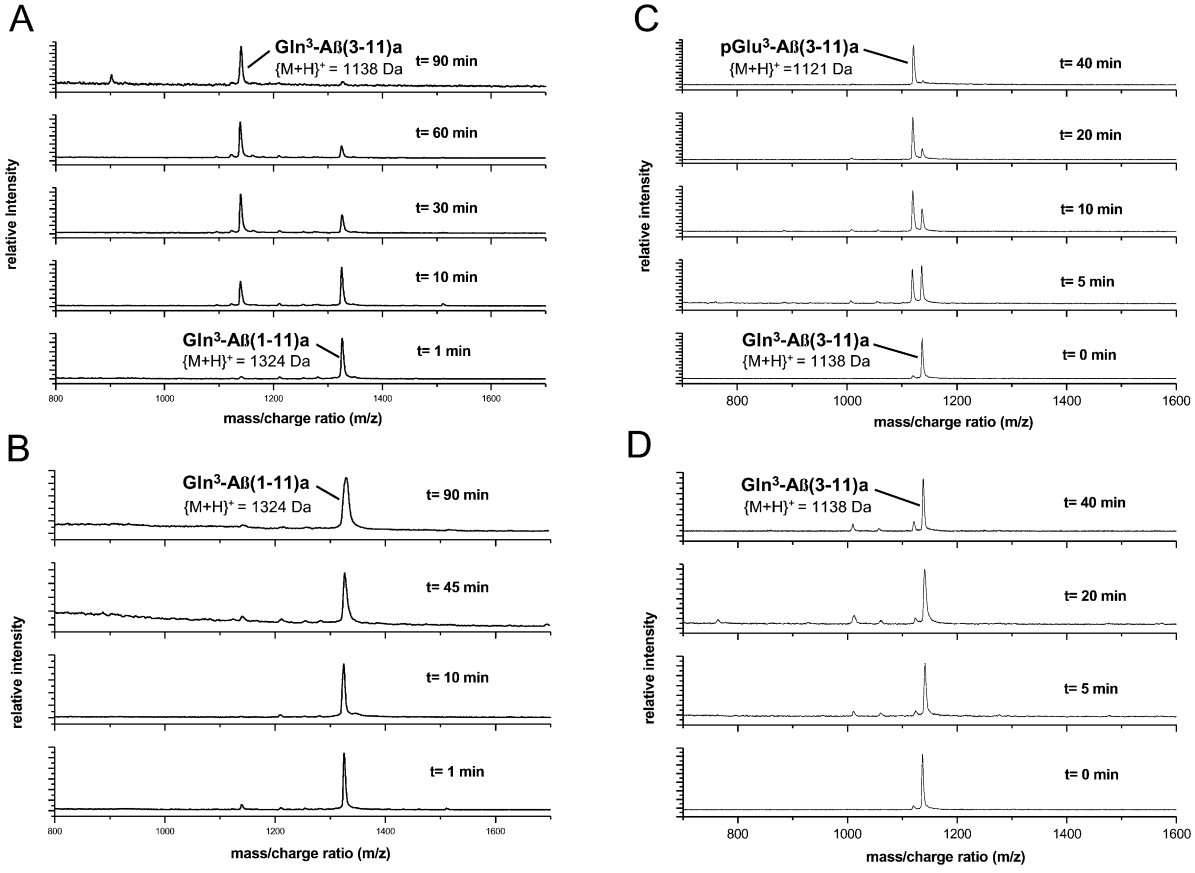


Fig. 1. A: Mass spectra of Gln^3-Aβ(1–11)a incubated with DPIV catalyzing the N-terminal truncation yielding Gln^3-Aβ(3–11)a. B: Mass spectra of Gln^3-Aβ(1–11)a incubated with DPIV and the DPIV inhibitor Val-pyrrolidide preventing N-terminal truncation of the peptide. C: Mass spectra of Gln^3-Aβ(3–11)a incubated with porcine pituitary homogenate catalyzing the formation of pGlu^3-Aβ(3–11)a. D: Mass spectra of Gln^3-Aβ(3–11)a incubated with QC and the QC inhibitor 1,10-phenanthroline preventing the formation of pGlu^3-Aβ(3–11)a.

analyzed fluorometrically [28]. For pH dependence studies the buffer contained 0.05 M acetic acid, 0.05 M pyrophosphoric acid and 0.05 M Tricine. Constant ionic strength and appropriate pH were maintained by addition of NaCl and NaOH, respectively. Enzyme kinetic data were analyzed using Grafit software (Erithacus Software).

2.4. MALDI-TOF mass spectrometry

Matrix-assisted laser desorption/ionization mass spectrometry was carried out using a Hewlett-Packard G2025 system. Reactions using the Gln peptides were performed in samples of 100 µl consisting of QC (0.01–1.0 U) and 0.5 mM substrate in 0.04 M Tris–HCl, pH 8.0, at 30°C or at pH and buffer conditions described below. At the times indicated, samples were removed, diluted with matrix and analyzed as described previously [27].

For long-term testing of Glu^1 cyclization, Aβ-derived peptides were incubated in 100 µl 0.1 M sodium acetate buffer, pH 5.2 or 0.1 M Bis-Tris buffer, pH 6.5 at 30°C. Peptides were applied in 0.5 mM Aβ(3–11)a and other synthetic peptides or 0.15 mM Aβ(3–21)a concentrations, and 0.2 U QC was added all 24 h. In the case of Aβ(3–21)a, the assays contained 1% dimethylsulfoxide (DMSO). At the times indicated in Figs. 1–3, samples were removed from the assay tube, peptides were extracted using ZipTips (Millipore), mixed with matrix solution (1:1 v/v) and subjected to mass spectrometry. Negative controls contained either no QC or heat-deactivated enzyme. For the inhibitor studies the sample composition was the same as described above, with the exception of the inhibitory compound added (5 mM benzimidazole or 2 mM 1,10-phenanthroline).

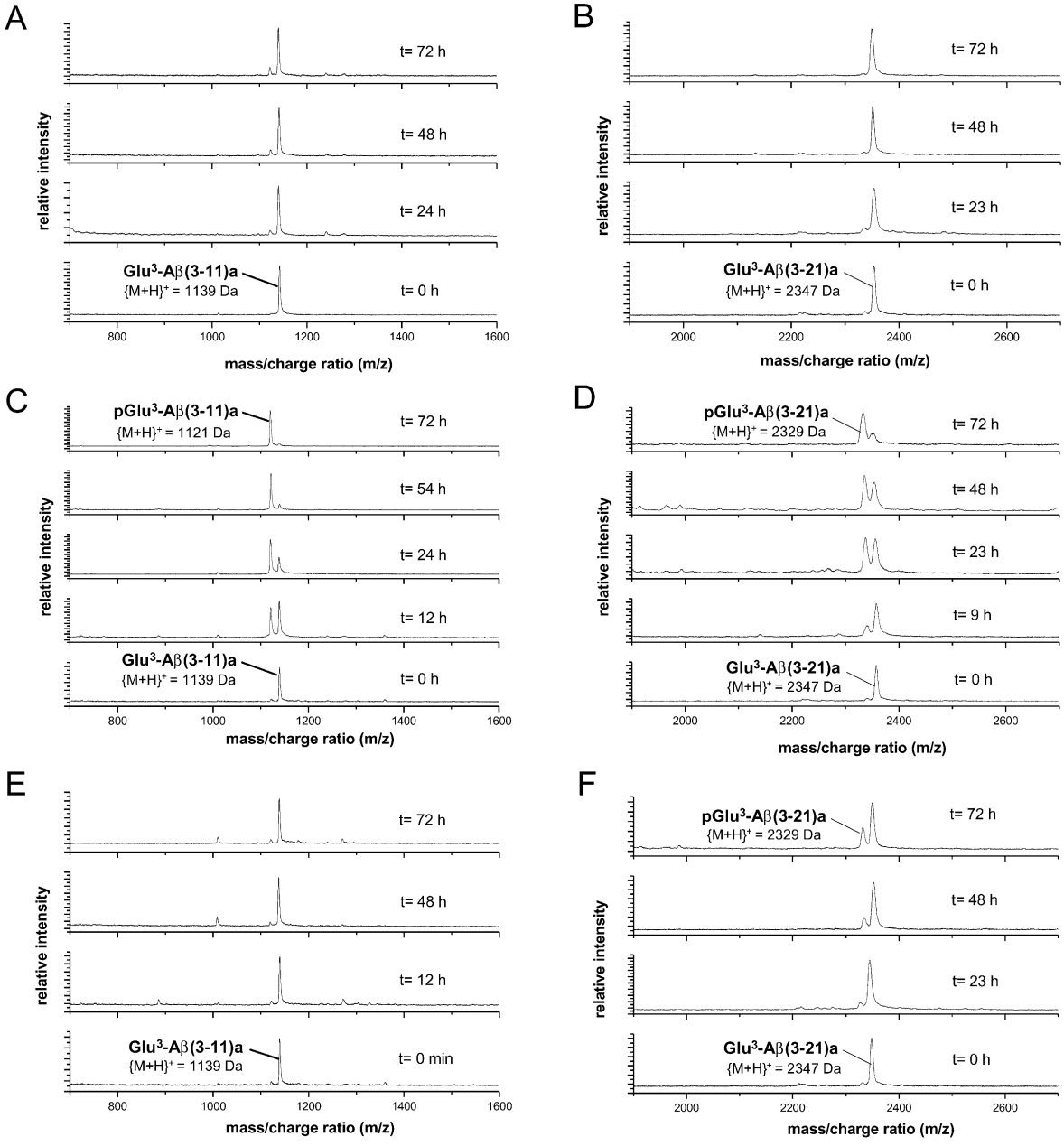


Fig. 2. A,B: Mass spectra of Glu^3-Aβ(3–11)a and Glu^3-Aβ(3–21)a incubated with recombinant human QC which was boiled for 10 min before use. C,D: Mass spectra of Glu^3-Aβ(3–11)a and Glu^3-Aβ(3–21)a in the presence of active human QC resulting in the formation of $pGlu^3$-Aβ(3–11)a and $pGlu^3$-Aβ(3–21)a, respectively. E,F: Mass spectra of Glu^3-Aβ(3–11)a and Glu^3-Aβ(3–21)a in the presence of active QC and 5 mM benzimidazole suppressing the formation of $pGlu^3$ formation.

3. Results

3.1. Turnover of Gln^3-Aβ peptides 3–11a, 3–21a and 3–40 by recombinant human QC

All Gln^3-Aβ-derived peptides tested were efficiently converted by human QC into the corresponding pGlu peptides (Table 1). Due to the poor solubility of Gln^3-Aβ(3–21)a and Gln^3-Aβ(3–40) in aqueous solution, 1% DMSO was added. The higher solubility of Gln^3-Aβ(3–11)a, however, allowed kinetic analysis in both the presence and absence of DMSO (Table 1). Taken together, the investigation of the Aβ peptides as QC substrates with chain lengths of 8, 18 and 37 amino acids (see Table 1) confirmed that human QC activity increases with the length of its substrates. Accordingly, Gln^1-gastrin, Gln^1-neurotensin, Gln^1-GnRH are among the best QC substrates [29]. Similarly, Gln^3-Aβ(3–40) and glucagon, the largest QC substrates investigated thus far, are cyclized highly specifically, even in the presence of 1% DMSO (Table 1). Therefore, due to better solubility and experimental handling, the further investigations concerning N-terminal processing of these peptides were performed using the smaller fragments of Aβ, Gln^3-Aβ(1–11)a, Gln^3-Aβ(3–11)a, Aβ(3–11)a and Aβ(3–21)a.

3.2. Processing of Gln^3-Aβ(1–11)a by purified DPIV and QC present in porcine pituitary homogenate

During posttranslational processing of APP, N-terminal degradation of the resulting Aβ peptide(s) occurs until the decomposition is halted by formation of N-terminal pGlu in vivo [16–22]. Since full-length Aβ(1–42) starts with the dipeptide Asp-Ala (a DPIV-recognition sequence) before Glu/Gln in position 3, we investigated whether purified DPIV or aminopeptidases of porcine pituitary homogenate were able to remove this sequence from our sample peptides. Incubation of the model peptides Gln^3-Aβ(1–11)a with DPIV and Gln^3-Aβ(3–11)a with porcine pituitary homogenate resulted in the formation of Gln^3-Aβ(3–11)a and pGlu^3-Aβ(3–11)a, respectively (see Fig. 1A,C). When the reaction was conducted in the presence of the DPIV inhibitor Val-pyrrolidide, no turnover of Gln^3-Aβ(1–11)a was observed (Fig. 1B). Similarly, in the presence of the QC inhibitor 1,10-phenanthroline, no final pGlu^3-Aβ(3–11)a formation occurred (Fig. 1D).

3.3. Turnover of Aβ(3–11)a and Aβ(3–21)a by recombinant human QC

The incubation of Aβ(3–11)a and Aβ(3–21)a in the presence of QC revealed that glutamate-containing peptides can also serve as QC substrates (Fig. 2C,D). The formation of pGlu^3-Aβ(3–11)a and pGlu^3-Aβ(3–21)a was investigated at pH 5.2 and 6.5, respectively. If the QC inhibitor benzimidazole was added to the solution before starting the assay by QC addition, substrate conversion was suppressed (Fig. 2E,F). If QC was boiled before addition, formation of the pGlu peptides was negligible (Fig. 2A,B). No turnover was detected at basic pH values.

Table 1
Kinetic parameters for conversion of N-terminally Gln-containing peptides by recombinant human QC in buffer solution containing 1% DMSO

Peptide	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})
Gln^3-Aβ(3–11)a	87 ± 3[a]	55 ± 1[a]	632 ± 10[a]
Gln^3-Aβ(3–11)a	155 ± 4	41.4 ± 0.4	267 ± 4
Gln^3-Aβ(3–21)a	162 ± 12	62 ± 3	383 ± 10
Gln^3-Aβ(3–40)	89 ± 10	40 ± 2	449 ± 28
Glucagon(3–29)	19 ± 1	10 ± 0.2	526 ± 17

[a]Determined in the absence of DMSO.

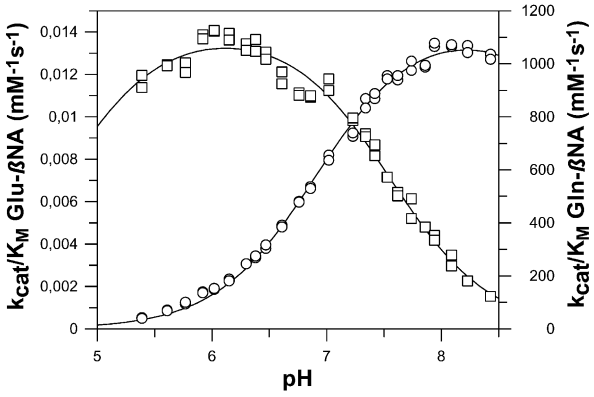


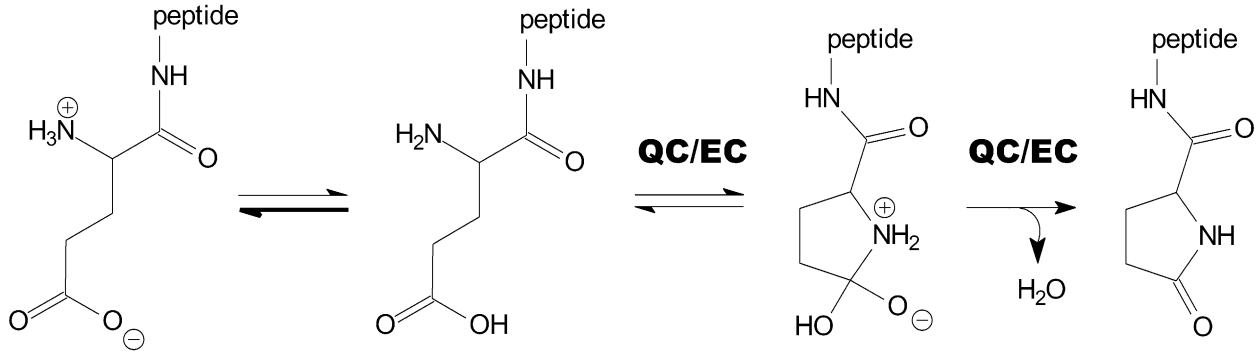
Fig. 3. pH dependence of the conversion of Gln-βNA (○) and Glu-βNA (□) by papaya QC, determined under first-order rate-law conditions ($S \ll K_M$). Substrate concentration were 0.01 mM and 0.25 mM, respectively. The data were fitted to equations that account for two dissociating groups revealing pK_a values of 6.91 ± 0.02 and 9.5 ± 0.1 for Gln-βNA and 4.6 ± 0.1 and 7.55 ± 0.02 for Glu-βNA. The pK_a values of the respective substrate amino groups, determined by titration, were 6.97 ± 0.01 (Gln-βNA) and 7.57 ± 0.05 (Glu-βNA).

3.4. pH dependence of the papaya QC-catalyzed cyclization of Gln-βNA and Glu-βNA

The plant QC from *C. papaya*, an analogous but non-homologous enzyme of the mammalian QCs, has been shown to possess similar catalytic properties to human QC, as revealed by the substrate specificity pattern and pH dependence of catalysis [29]. Accordingly, plant QC cyclized Aβ(3–11)a at pH 5.2 (not shown). In contrast to human QC, however, it readily converts the fluorogenic substrate Glu-βNA allowing a coupled fluorometric assay [28]. Papaya QC converted Glu-βNA in a concentration range up to 2 mM in accordance with Michaelis–Menten kinetics (not shown). Hence, papaya QC was used to study the pH dependence of the conversion of Glu-βNA and Gln-βNA. These model reactions were investigated under first-order rate-law conditions using higher enzyme amounts (Fig. 3). As expected the cyclization of glutamine has a pH optimum at pH 8.0. In contrast, the cyclization of glutamic acid showed a pH optimum of pH 6.0. While the specificity constants at the respective pH optima differ approximately 80 000-fold, the ratio of QC versus glutamyl cyclase (EC) activity around pH 6.0 is only about 8000. The non-enzymatic pGlu formation from Gln-βNA, investigated at pH 6.0, was followed for 4 weeks and revealed a first-order rate constant of 1.2×10^{-7} s^{-1}. However, during the same time period, no pGlu-βNA was formed from Glu-βNA enabling the estimation of a limiting rate constant for turnover of 1.0×10^{-9} s^{-1}.

4. Discussion

Since formation of pGlu^1 peptides such as pGlu^3-Aβ(3–40) occurs neither in vitro nor in vivo spontaneously, only the enzymatic cyclization of e.g. Glu-Aβ by a putative EC is con-



Scheme 2. N-terminal cyclization of uncharged glutamyl peptides by QC (EC).

ceivable. Here, we have shown that papaya and human QC catalyze both glutaminyl and glutamyl cyclization (Schemes 1 and 2, Figs. 1–3).

Investigating the pH dependence, we found the unprotonated N-terminus essential for the cyclization of Gln^1 peptides. Accordingly, the pK_a value of the substrate was identical to the pK_a value for QC catalysis (see Fig. 3 and [29]), supporting that QC stabilizes the nucleophilic attack of the unprotonated α-amino moiety on the amidated γ-carbonyl carbon (Scheme 1). In contrast to the monovalent charge present on N-terminal glutamine-containing peptides, the N-terminal Glu residue in Glu-containing peptides is predominantly bivalently charged around neutral pH. Glutamate exhibits pK_a values of about 4.2 and 7.5 for the γ-carboxylic and the α-amino moiety, respectively; i.e. at neutral pH and above, although the α-amino nitrogen is partially or fully unprotonated and nucleophilic, the γ-carboxylic group is negatively charged, so exercising no electrophilic carbonyl activity. However, in the pH range of about 5.2–6.5, between their respective pK_a values, the two groups are present both partially non-ionized, in concentrations less than 10% of the total N-terminal Glu-containing peptide; i.e. if the γ-carboxylic group is protonated, the carbonyl carbon is electrophilic enough to allow nucleophilic attack by the unprotonated α-amino group. According to the Henderson–Hasselbalch equation, the concentration of the uncharged molecule species is about 0.05% of the total substrate at pH 6.0. Only in these molecules the hydroxyl ion can serve as a leaving group (Scheme 2). These assumptions are corroborated by the pH dependence data obtained for the QC-catalyzed conversion of Glu-βNA. In contrast to glutamine conversion of Gln-βNA by QC, the pH optimum of catalysis shifts to the acidic range. Furthermore, the kinetically determined pK_a value of 7.55 ± 0.02 is in excellent agreement with that of the α-amino group of Glu-βNA, determined by titration (7.57 ± 0.05).

There was no non-enzymatic turnover of Glu-βNA, which agrees with the observed negligible pGlu peptide formation. The conclusion from these data is that in vivo only enzymatic transformation of N-terminal Glu seems conceivable. In vitro, applying inhibitors of QC and EC activity, we were able to suppress both reactions (Figs. 1 and 2).

Physiologically, Aβ can be generated either directly on the plasma membrane or during endosomal recycling or in the endoplasmic reticulum (ER), the *trans*-Golgi network or within secretory vesicles [30–32]. Recently, the constitutive secretory pathway providing Aβ has been discussed [33]. Coincidentally, QC is also localized in the secretory pathway [4–7]. Although Glu-Aβ peptides have been found to be generated in the ER, the Golgi network and within secretory vesicles, further detailed cell biological work is needed to identify the subcellular site of pGlu-Aβ peptide formation.

The primary biological function of QC is likely terminal hormone maturation in endocrine cells by glutamine cyclization during the hormone secretion process. Such secretory vesicles are known to be acidic in pH. Thus, an auxiliary function of the enzyme in the narrow pH range from 5.0 to 7.0 could be its glutamyl cyclase activity (Scheme 2). However, due to the relatively inefficient rate of Glu cyclization compared to Gln conversion, it is questionable whether glutamyl cyclization plays a significant physiological role. In the etiology of neurodegenerative disorders, however, glutamyl cyclization may be of relevance providing that accumulation of peptidase-resistant substrate and appropriate QC concentration and compartment acidity coincide.

Reviews of potential AD treatment do not propose inhibition of pyroglutamate formation as potential therapeutic intervention [9,13,34]. Since pGlu-Aβ peptides appear highly abundant, and since pGlu-Aβ formation prevents intracellular aminopeptidase-mediated disposal of such improperly generated peptides [30,35], inhibition of brain QC and EC activity could prove a valuable tool to combat the onset and progression of neurodegenerative disorders.

Whether N-terminal processing of Aβ-derived peptides takes place in vivo by a combination of aminopeptidase, dipeptidyl peptidase and glutaminyl/glutamyl cyclase activity needs further investigation. Previously, similar N-terminal processing in an analogous fashion was suggested for the neuropeptide antho-RFamide precursors of coelenterates [36].

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