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Heterologous Expression and Characterization of Human Glutaminyl Cyclase: Evidence for a Disulfide Bond with Importance for Catalytic Activity

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ABSTRACT: Glutaminyl cyclase (QC, EC 2.3.2.5) catalyzes the formation of pyroglutamate residues from glutamine at the N-terminus of peptides and proteins. In the current study, human QC was functionally expressed in the secretory pathway of Pichia pastoris, yielding milligram quantities after purification from the supernatant of a 5 L fermentation. Initial characterization studies of the recombinant QC using MALDI-TOF mass spectrometry revealed correct proteolytic processing and N-glycosylation at both potential sites with similar 2 kDa extensions. CD spectral analysis indicated a high α-helical content, which contrasts with plant QC from Carica papaya. The kinetic parameters for conversion of H-Gln-Tyr-Ala-OH by recombinant human QC were almost identical to those previously reported for purified bovine pituitary QC. However, the results obtained for conversion of H-Gln-Gln-OH, H-Gln-NH₂, and H-Gln-AMC were found to be contradictory to previous studies on human QC expressed intracellularly in E. coli. Expression of QC in E. coli showed that approximately 50% of the protein did not contain a disulfide bond that is present in the entire QC expressed in P. pastoris. Further, the enzyme was consistently inactivated by treatment with 15 mM DTT, whereas deglycosylation had no effect on enzymatic activity. Analysis of the fluorescence spectra of the native, reduced, and unfolded human QC point to a conformational change of the protein upon treatment with DTT. In terms of the different enzymatic properties, the consequences of QC expression in different environments are discussed.

Besides proteolytic cleavage and C-terminal amidation, N-terminal formation of 5-oxoproline (pyroglutamate, pGlu) is a common post-translational event during the biosynthesis of a number of peptides. Examples of glutaminate containing peptides include the hormones thyrotropin-releasing hormone (TRH) and gastrin, the neuropeptide neurotensin, and the chemokines MCP-1 through MCP-4 (1, 2). For peptides such as TRH and MCP-2, biological function has been shown to depend on the 5-oxoproline at their N-terminus. Loss or modification of this residue leads to a decrease in biological activity (3, 4). The maturation of these peptide hormones, taking place in the regulated secretory pathway (RSP), is well understood, and many of the enzymes involved in the pro-hormone to hormone conversion have been identified and characterized (5, 6). The enzyme glutaminyl cyclase (QC), however, responsible for formation of pyroglutamate from glutamine at the N-termini of hormones, is poorly understood.

First identified in the plant Carica papaya (7), QCs have been reported from a bovine (8), porcine (9), and human as well as other mammalian sources (10, 11). Though the QCs from plants and mammals are similar in size (33 kDa and 38–40 kDa respectively), recent studies have revealed little or no sequence homology between them (12). A highly conserved primary structure, however, was reported for QCs from different mammalian species (11).

Due to the abundance of QC in papaya latex and to a simple isolation procedure, the majority of biochemical data of QC has been collected for the papaya enzyme (13–15). To date, there has been only one report of QC purification to homogeneity from a natural mammalian source. On the basis of considerable effort, 38 μg of homogeneous QC could be recovered from 2000 bovine pituitaries (16). Subsequently, cDNAs encoding the bovine and human enzyme, respectively, have been isolated, and enzymological studies with recombinant human QC expressed intracellularly in Escherichia coli have been reported (10, 11). Aggregation of the protein during bacterial expression, however, has necessitated protein refolding under denaturing conditions or expression of the QC as a fusion protein (N-terminal mannose binding protein or glutathione S-transferase) in order to recover the active protein.

These difficulties tempted us to express human QC in another host system, Pichia pastoris. This methylotrophic
yeast shares the advantages of bacterial hosts, such as simple genetic manipulation, simple culture conditions and rapid growth, while facilitating post-translational modification in a manner more similar to that of higher eukaryotes (e.g., N-glycosylation, disulfide formation, fatty acylation and C-terminal methylation). Further, P. pastoris allows high-level expression of heterologous proteins either intracellularly or in secreted form (17–19), as well as allowing simple scale-up production using a fermenter.

Here, we describe the large-scale expression of human QC in P. pastoris and the subsequent identification of important enzymatic properties in contrast to those obtained from human QC expressed in E. coli.

EXPERIMENTAL PROCEDURES

Host strains and media. The E. coli strain JM109 was applied for all plasmid constructions and propagation. P. pastoris strain X33 (AOX1, AOX2), used for the expression of human QC was grown, transformed, and analyzed according to the manufacturer’s instructions (Invitrogen). Media for propagation of E. coli, i.e., low salt LB, as well as the media required for P. pastoris, i.e., buffered glycerol (BMGY) complex or methanol (BMMY) complex medium, and the fermentation basal salts medium were prepared according to the manufacturer’s recommendations.

Isolation of QC cDNA and site-directed mutagenesis. A full-length cDNA encoding the human QC was isolated from clone DKFZp566F243, obtained from the resource center of the human genome project at the Max-Plank-Institute for Molecular Genetics (Berlin, Germany). Sequencing (Seqlab GmbH, Göttingen) of the cDNA revealed three single base exchanges in the open reading frame, one in codon 15 replacing CTG (Leu) by CCG (Pro), a silent exchange in codon 98 (CTC instead of CTT), and in codon 164 TGT (Cys) was replaced by TGG (Trp). Site-directed mutagenesis was carried out to replace Trp with Cys at position 164. All exchanges in the open reading frame, one in codon 15 replacing CTG (Leu) by CCG (Pro), and the fermentation basal salts medium were prepared according to the manufacturer’s recommendations.

Molecular cloning of plasmids vectors encoding the human QC. All cloning procedures were done applying standard molecular biology techniques (20). For expression in yeast, the vector pPICZaB (Invitrogen) was used that covers a coding sequence for the S. cerevisiae α-factor prepro-peptide upstream of a multiple cloning site. To express the QC with an N-terminal 6× histidine affinity tag, a cassette consisting of the oligonucleotides HPic-K1 and HPic-K2 (Table 1) was inserted in frame with the leader sequence using the restriction sites PstI and KpnI. In addition, with the cassette a novel restriction site for Nhel was introduced. Finally, the cDNA encoding the mature QC starting with amino acid 33 was amplified by PCR using the primers QC−Pic1 an QC−Pic2 (Table 1). Subsequently, subcloning into the pCRScript Cam SK (+) vector and insertion into the yeast expression plasmid via the Nhel and XbaI restriction sites was performed.

The pQE−31 vector (Qiagen) was used to express the human QC in E. coli. The cDNA of the mature QC starting with codon 38 was fused in frame with the plasmid encoded 6× histidine tag. After amplification utilizing the primers pQCyc−1 and pQCyc−2 (Table 1) and subcloning, the fragment was inserted into the expression vector employing the restriction sites of Spfh and HindIII. All expression plasmids were sequenced using either vector- or cDNA-specific primers.

Transformation of P. pastoris and mini-scale expression. Plasmid DNA was amplified in E. coli JM109 and purified according to the recommendations of the manufacturer (Qiagen). In the expression plasmid used, pPICZaB, three restriction sites are provided for linearization. Since SacI and BsrXI cut within the QC cDNA, PmelI was chosen for linearization. 20–30 μg plasmid DNA was linearized with PmelI, precipitated by ethanol, and dissolved in sterile, deionized water. A 10 μg sample of the DNA was then applied for transformation of competent P. pastoris cells by electroporation according to the manufacturer’s instructions (BioRad). Selection was done on plates containing 150 μg/mL Zeocin. One transformation using the linearized plasmid yielded several hundred transformants.

To test the recombinant yeast clones for QC expression, recombinants were grown for 24 h in 10 mL conical tubes containing 2 mL BMGY. Afterward, the yeast was centrifuged and resuspended in 2 mL BMMY containing 0.5% methanol. This concentration was maintained by addition of methanol every 24–72 h. Subsequently, QC activity in the supernatant was determined. The presence of the fusion protein was confirmed by western blot analysis using an

Table 1: Oligonucleotides Used in Cloning Procedures for Heterologous Expression of QC in E. coli and P. pastoris

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>sequence (5’→3’), restriction sites (underlined)</th>
<th>restriction enzyme for cloning</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC−SDMCs</td>
<td>GCGCTGCCCATGTCAGAATGTTG</td>
<td>−</td>
</tr>
<tr>
<td>QC−SDMCas</td>
<td>CAACATATGAGGACATGGAAC</td>
<td>−</td>
</tr>
<tr>
<td>QC4</td>
<td>ATAGTGGGAGGCGAGAGAGCC</td>
<td>Safl</td>
</tr>
<tr>
<td>QC5</td>
<td>ATAGCTACCTTACAAATGAGATATC</td>
<td>HindIII</td>
</tr>
<tr>
<td>pQCyc−1</td>
<td>ATATAGCTGGAGGAGAGAGAAATACACCAG</td>
<td>Spfh</td>
</tr>
<tr>
<td>pQCyc−2</td>
<td>ATATAAGCCTCAAATGAGATATCCAAC</td>
<td>HindIII</td>
</tr>
<tr>
<td>QC−Pic1</td>
<td>ATGCTAGCGCTGGACAGGAGAGAAATCG</td>
<td>Nhel</td>
</tr>
<tr>
<td>QC−Pic2</td>
<td>ATTCTAGATATTACAATGAGATATCC</td>
<td>Nhel</td>
</tr>
<tr>
<td>HPic−K1</td>
<td>CGTCATCATCATCATCATCATGCTGCGGCT</td>
<td>Nhel</td>
</tr>
<tr>
<td>HPic−K2</td>
<td>CGCTAGCATGATGATGATGATGATGATGATGCTGCA</td>
<td>Nhel</td>
</tr>
</tbody>
</table>
antibody directed against the 6×histidine tag (Qiagen). Clones that displayed the highest QC activity were chosen for further experiments and fermentation.

**Large-scale expression in a fermenter.** Expression of the QC was performed in a 5 L reactor (Biostad B, B. Braun biotech), essentially as described in the "Pichia fermentation process guidelines" (Invitrogen). In brief, the cells were grown in the fermentation basal salts medium supplemented with trace salts, and with glycerol as the sole carbon source (pH 5.5). During an initial batch phase for about 24 h and a subsequent fed-batch phase for about 5 h, cell mass was accumulated. Once a cell wet weight of 200 g/L was achieved, induction of QC expression was performed using methanol applying the three-step feeding profile recommended by invitrogen for an entire fermentation time of approximately 60 h. Subsequently, cells were removed from the QC-containing supernatant by centrifugation at 6000 g, 4 °C for 15 min. The pH was adjusted to 6.8 by addition of NaOH, and the resultant turbid solution was centrifuged at 37000 g, 4 °C, for 40 min. In cases of continued turbidity, an additional filtration step was applied using a cellulose membrane (pore width 0.45 μm).

**Purification of 6×histidine tagged QC expressed in P. pastoris.** The His-tagged QC was first purified by immobilized metal ion affinity chromatography (IMAC). In a typical purification, 1000 mL of culture supernatant was applied to a Ni2+-loaded Chelating Sepharose FF column (1.6 × 20 cm, Pharmacia) that was equilibrated with 50 mM phosphate buffer, pH 6.8, containing 750 mM NaCl, at a flow rate of 5 mL/min. After washing with 10 column volumes of equilibration buffer and 5 column volumes of equilibration buffer containing 5 mM histidine, the bound protein was eluted by a shift to 50 mM phosphate buffer, pH 6.8, containing 150 mM NaCl and 100 mM histidine. The resulting eluate was dialyzed against 20 mM Bis-Tris/HCl, pH 6.8, at 4 °C overnight. Subsequently, the QC was further purified by anion exchange chromatography on a Mono Q6 column (BioRad), equilibrated with dialysis buffer.

The QC-containing fraction was loaded onto the column using a flow rate of 4 mL/min. The column was then washed with equilibration buffer containing 100 mM NaCl. The elution was performed by two gradients, resulting in equilibration buffer containing 240 and 360 mM NaCl in 30 or 5 column volumes, respectively. Fractions of 6 mL were collected and the purity was analyzed by SDS–PAGE. Fractions containing homogeneous QC were pooled and concentrated by ultrafiltration. For long-term storage (−20 °C), glycerol was added to a final concentration of 50%. Protein was quantified according to the methods of Bradford or Gill and von Hippel (21, 22).

**Expression and purification of QC in E. coli.** The construct encoding the QC was transformed into M15 cells (Qiagen) and grown on selective LB agar plates at 37 °C. Protein expression was carried out in LB medium containing 1% glucose and 1% ethanol at room temperature. When the culture reached an OD600 of approximately 0.8, expression was induced with 0.1 mM IPTG overnight. After one cycle of freezing and thawing, cells were lysed at 4 °C by addition of 2.5 mg/mL lysozyme in 50 mM phosphate buffer, pH 8.0, containing 300 mM NaCl and 5 mM histidine for approximately 30 min. The solution was clarified by centrifugation at 37000 g, 4 °C for 30 min, followed by two filtration steps applying cellulose filters for crude and fine precipitates and an additional filtration using a regenerated cellulose membrane (0.45 μm pore width). The supernatant was applied onto the Ni2+-affinity column according to the purification of QC expressed in P. pastoris. In contrast to the aforementioned preparation, one additional washing step with equilibration buffer containing 15 mM histidine was implemented. Elution of QC was carried out with 50 mM phosphate buffer containing 150 mM NaCl and 100 mM histidine. The QC-containing fraction was concentrated by ultrafiltration and immediately used for further experiments or stored as described for the QC expressed in P. pastoris.

**Synthesis of H-Gln-Tyr-Ala-OH and H-Gln-His-Pro-NH2.** Semi-automated synthesis of the tripeptides was performed on a 0.5 mmol scale using a peptide synthesizer (Labortec SP650) and the standard Fmoc-protocol of solid-phase peptide synthesis. Cycles were modified by using double couplings (shaking 2 × 24 min) with a 2-fold excess of Fmoc-Tyr(tBu)-OH or Fmoc-His(Trt)-OH and Fmoc-Gln-(Trt)-OH, employing the preloaded Fmoc-Ala-Wang (substitution 1.1 mmol/g) in case of H-Gln-Tyr-Ala-OH or the Rink Amide MBHA resin (substitution 0.79 mmol/g) in case of H-Gln-His-Pro-NH2. The Wang resin was preloaded in our laboratories according to standard procedures. Fmoc deprotection was carried out by using 20% piperidine in dimethylformamide (1 × 3 min, 1 × 7 min). The amino acid couplings were performed by 2-((1H-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium tetrafluoroborate (TBTU) (2 equiv)/diisopropyl ethylamine (4 equiv) activation in dimethylformamide. Cleavage from the resin was accomplished with a cocktail consisting of 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisopropylsilane and yielded 80% of the crude peptide containing approximately 4% of the pyroglutamyl peptide. The crude peptides were precipitated by cold ether and separated from the pyroglutamyl peptide by preparative HPLC with TFA free solvents in order to avoid further cyclization of the N-terminal glutamine. Preparative HPLC was performed with a linear gradient of acetonitrile in water (5–65% acetonitrile over 40 min) on a 250–21 Luna RP18 column. Lyophilization resulted in a white, fluffy substance. To confirm peptide purity and identity, analytical HPLC and ESI-MS were employed. CHN analysis of H-Gln-Tyr-Ala-OH was consistent with the glutaminyl peptide containing one molecule of TFA and one molecule of water per molecule of peptide.

**Assays for glutaminyl cyclase activity.** All measurements were performed with a BioAssay Reader HTS-7000Plus for microplates (Perkin-Elmer) at 30 °C. QC activity was evaluated fluorometrically using H-Gln-β-Na, essentially as described (23). The samples consisted of 0.2 mM fluorogenic substrate and 0.25 U pyroglutamyl aminopeptidase (Unizyme, Hørsholm, Denmark) in 0.2 M Tris/HCl, pH 8.0, containing 20 mM EDTA (50 mM Tris/HCl, pH 8.0, containing 5 mM EDTA in case of determination of the kinetic parameters) and 40–400 ng QC in a final volume of 250 μl. Excitation/emission wavelengths were 320/410 nm. The assay reactions were initiated by addition of glutaminyl cyclase. QC activity was determined from a standard curve of β-naphthylamide under assay conditions. One unit is defined as the amount of QC catalyzing the formation of 1 μmol pGlu-β-Na from H-Gln-β-Na per minute under the described conditions.
Spectral analysis of QC and investigation of the disulfide bond status. CD spectra of human QC in 20 mM phosphate buffer, pH 6.8, were acquired with a Jasco J-715 spectropolarimeter using quartz cuvettes of 0.1 cm path length. The mean of 15 scans between 185 and 250 nm was calculated and corrected by subtraction of the buffer spectra. The percentage of secondary structure elements was calculated and corrected by subtraction of the buffer spectra. The proteolytic processing results in a secreted, 6×histidine tagged QC, starting with alanine 33 of the native sequence.

RESULTS

Expression of human QC in P. pastoris. As demonstrated for QC from bovine pituitary and hypothalamus, the enzyme is directed to the secretory pathway caused by an N-terminal leader sequence and was colocalized in the secretory granules with its products of catalysis, e.g., TRH (16, 28). Therefore, secretory expression should also be employed when QC is produced in P. pastoris. The N-terminal leader sequences of proteins of higher eucaryotes often yield only small quantities of secreted proteins in yeast. To improve the secretion efficiency in P. pastoris, the mature QC cDNA was fused to the plasmid-encoded sequence of the α-factor prepro-peptide from S. cerevisiae, capable of directing proteins efficiently to the secretory pathway (29). This sequence is extended by codons for two Glu–Ala and for one Ala–Ala that are post-translationally cleaved by dipeptidyl aminopeptidase A (DAP A). In addition, the Glu–Ala repeats are thought to favor the cleavage of the α-factor by Kex2. A coding sequence of a 6×histidine tag was attached to the 5′-end of the mature QC cDNA in order to facilitate the purification. Thus, expression of the construct should result in a secreted protein with 6 histidine residues at its N-terminus followed by mature QC starting with amino acid alanine 33, as illustrated in Figure 1.

To increase the integration frequency of the construct for overexpression, plasmids were linearized in the sequence of the AOX1 promoter by an endonuclease treatment using the Pmel restriction site. Initially, transformants were checked for integration by PCR, immunodetection, and activity measurements. Since all clones tested were positive, additional recombinants were only checked by QC activity evaluation. Finally, three of 100 clones showing the highest expression level were chosen for scaled-up expression in a fermenter.

A typical fermentation procedure is documented in Fig. 2 by the time course of the optical density at 600 nm (OD600), cell weight and QC activity appearing in the fermentation medium. The fermentation consisted of the three stages glycerol batch, glycerol fed batch, and methanol fed batch. The glycerol phases were marked by rapid growth of the yeast cells but lacked any QC activity. The production phase, starting upon depletion of glycerol and supply of methanol, was indicated by decreased cell growth and appearance of first QC activity, indicating that QC expression depended on activation of the AOX promoter. The enzymatic activity increased throughout the fermentation.
Upon initial purification of the QC-containing fractions by affinity chromatography on immobilized nickel ions (IMAC), there were still apparent impurities by a protein of about 2 kDa less than the QC-containing band. Since impurities increased during fermentation, represented by an increase in this lower band, and appearance of a third band that became the sole band after 96 h of fermentation, the QC was purified from cultures grown after 60 h of fermentation. At this stage, only residual impurities were found (Figure 3A). Further purification was performed by chromatography using a strong anion-exchange resin and a very broad salt gradient. Despite a surprisingly small yield, 4 mg of pure QC was ultimately obtained from the 5 L fermentation. The purification procedure is shown in Figure 3B and Table 2.

Characterization of human QC expressed in P. pastoris. Western blot analysis following IMAC revealed that QC and both impurities contained a histidine tag (data not shown). Since the lowest band of the three species could be separated by lectin affinity chromatography (data not shown), two of the three protein species seemed to be less glycosylated forms of QC. This conclusion was corroborated by MALDI-TOF mass spectrometry. The recombinant human QC displayed a relatively broad peak at a molecular mass of 42.8 kDa (Figure 4A). Upon deglycosylation with endoglycosidase Hf, two other protein species exhibiting molecular masses of 41.1 kDa and 38.8 kDa were formed consecutively (Figure 4B–E). The primary structure of QC reveals two potential N-glycosylation sites, located at asparagine residues 49 and 296 (10). Thus, in the recombinant QC, both asparagines were glycosylated with oligosaccharides of about 2 kDa per residue, suggesting that QC is also expressed as a glycoprotein in mammalian cells.

Using MALDI-TOF mass analysis with bovine serum albumin as an internal standard, from five independently recorded mass spectra, a molecular mass of 38,795 ± 19 Da was determined for the quantitatively deglycosylated enzyme (not shown). This mass corresponds well to the theoretical value of 38,745 Da, calculated from all amino acid residues and the two GlcNAc residues remaining after deglycosylation by endoglycosidase Hf. Therefore, post-translational modifications other than N-glycosylation are
improbable during expression, and the N-terminus seems to be completely processed by Kex2 and DAP A, a frequent cause of inhomogeneties observed when foreign genes are expressed in *P. pastoris*.

Further characterization of recombinant human QC was performed by applying CD spectroscopy (Figure 5). The appearance of the spectrum indicates a dominant α-helix content. The two minima at 208 and 222 nm are characteristic for proteins that contain a high portion of α-helix in their overall secondary structure. A calculation of quantities of α-helix, β-sheet, turn, and random structure revealed an α-helix content of 47% for the human QC. This amount contrasts with the 5% content reported for the QC from papaya latex, indicating completely different folding patterns for both proteins.

Kinetic parameters were recorded for the recombinant human QC in order to characterize the catalytic competence of the enzyme. The values obtained at 30 °C with the substrates H-Gln-Tyr-Ala-OH, H-Gln-His-Pro-NH₂, H-Gln-AMC, H-Gln-βNA, H-Gln-NH₂, and H-Gln-Gln-OH as substrates are listed in Table 3. Upon examination at 37 °C, the kinetic parameters *Kₘ* and *kₐ₅* for conversion of H-Gln-Tyr-Ala-OH shifted to 153 ± 5 μM and 220 ± 2 s⁻¹, respectively.

Interestingly, the data found are in good agreement with values determined with QC from papaya latex. For instance, H-Gln-AMC and H-Gln-βNA were converted with *Kₘ* values of 52 ± 5 μM and 43 ± 4 μM and *kₐ₅* values of 31 and 46 s⁻¹, respectively (23). The kinetic parameters listed in Table 3 are in striking contrast, however, to those found by Song et al. for human QC, expressed in *E. coli* (10). In this study, a *Kₘ* value of 0.64 mM and a *kₐ₅* of 50.9 min⁻¹ were obtained for conversion of H-Gln-Gln-OH. Also, H-Gln-NH₂ was processed differently. The enzyme expressed in *P. pastoris* exhibits approximately 3-fold tighter binding and 30-fold faster turnover compared to recombinant human QC expressed in *E. coli* (30). Most strikingly, H-Gln-AMC was not converted at all by the human QC expressed in *E. coli* (10), while the recombinant human QC from *P. pastoris* cyclized H-Gln-AMC almost comparable to other substrates (Table 3).

Recombinant expression and characterization of human QC in *E. coli*. The remarkably different kinetic properties of human QC expressed in *P. pastoris* with that formerly described for QC expressed in *E. coli* (10) prompted a more detailed comparison. The cDNA starting with codon 38 was cloned into the pQE-31 vector and expressed in the cytosol with an N-terminal 6× histidine tag. Minimal enzymatic activity was detected upon expression at 37 °C using LB broth and induction with 0.1 mM IPTG for 5 h. After overnight expression at room temperature in LB medium supplemented with 1% glucose and 1% ethanol, however, a 50-fold increase in QC activity was found. Supernatants of enzymatically lysed bacteria were clarified by several centrifugation and filtration steps and the resulting soluble QC was purified to apparent homogeneity by IMAC in one step (Figure 6). Usually, about 5 mg QC could be purified per 2 L culture corresponding to an overall yield of 20%.

Applying H-Gln-βNA as substrate, a *Kₘ* value of 62 ± 5 μM and a *kₐ₅* of 7.5 ± 0.3 s⁻¹ were found. Thus, compared to the human QC expressed in yeast, the enzyme expressed in *E. coli* showed an identical *Kₘ* value, but an approximately 3-fold lower turnover number. A frequent reason for reduced or abolished activity of proteins expressed heterologously in *E. coli* is the lack of post-translational modifications such as proper disulfide bond formation or N-glycosylation. Therefore, the presence of disulfide bonds in the recombinant QCs and the influence of glycosylation on the catalysis were tested. Interestingly, deglycosylation by endoglycosidase H₄ did not alter the kinetic parameters of QC expressed in yeast using Gln-βNA as substrate, indicating that the lower activity was not caused by lack of glycosylation (not shown). QC contains only two cysteine residues, one in position 139 and another one in 149. Thus, the disulfide status could be easily analyzed. In a one-dimensional SDS–PAGE after reducing and nonreducing sample preparation, disulfide-containing polypeptides migrate different from their reduced counter-

**Figure 5:** CD-spectroscopic analysis of the secondary structure of recombinant human QC. The protein was dissolved in 20 mM potassium phosphate buffer, pH 6.8. Estimation of the secondary structure revealed 47% α-helix, 14% β-sheet, and 14% β-turn content.

**Figure 6:** SDS–PAGE of the purification steps of human QC expressed in *E. coli*. Lanes: 1, molecular mass standards (kDa); 2, supernatant after lysis, 3, purified QC after IMAC. Electrophoresis was performed in 12% gels using reducing sample preparation as described elsewhere (41).

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**Table 3: Kinetic Parameters Determined for Human QC Expressed in *P. pastoris*²**

<table>
<thead>
<tr>
<th>QC substrate</th>
<th>Michaelis constant (μM)</th>
<th>turnover number (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-Gln-Tyr-Ala-OH</td>
<td>101 ± 4</td>
<td>125 ± 1</td>
</tr>
<tr>
<td>H-Gln-His-Pro-NH₂</td>
<td>90 ± 4</td>
<td>83 ± 1</td>
</tr>
<tr>
<td>H-Gln-AMC</td>
<td>51 ± 3</td>
<td>5.4 ± 0.1</td>
</tr>
<tr>
<td>H-Gln-βNA</td>
<td>60 ± 6</td>
<td>18.8 ± 0.7</td>
</tr>
<tr>
<td>H-Gln-NH₂</td>
<td>409 ± 40</td>
<td>12.8 ± 0.5</td>
</tr>
<tr>
<td>H-Gln-Gln-OH</td>
<td>148 ± 5</td>
<td>20.7 ± 0.2</td>
</tr>
</tbody>
</table>

² Reactions were carried out at 30 °C in 0.05 M Tris/HCl, pH 8.0, containing 5 mM EDTA.
parts, indicating the presence of intramolecular disulfide bonds (26). In the case of QC expressed in yeast and separated by SDS–PAGE after nonreducing sample preparation (Figure 7A, lanes 4–7), the protein clearly migrated faster than that after a reducing sample preparation (lanes 2 and 3 and lanes 8 and 9), providing evidence of a disulfide bond in native human QC. In contrast, two bands of similar strength were formed after nonreducing sample preparation in the case of QC expressed in E. coli (Figure 7B, lanes 4–7). This clearly indicates that not all of the QC expressed in E. coli contained a disulfide bond. Accordingly, color development using Ellman’s reagent was only detected in the case of the QC that was expressed in E. coli. The portion of the protein being free of a disulfide bond was calculated to be 50% using an absorption coefficient of 13 600 M⁻¹ cm⁻¹ (27).

Effects of disulfide reduction on activity and structure of QC expressed in P. pastoris. Due to the obvious effect of the disulfide on the active structure of QC, the influence of reducing agents was examined. In the absence of a reducing agent, QC activity was constant at pH 6.8 and room temperature for 2 h (Figure 8). In contrast, QC was readily inactivated by 15 mM dithiotreitol (DTT) during this time. The loss of QC activity appeared exponentially and was independent of the initial amount, suggesting pseudo-first-order kinetics of inactivation. A shift of the pH from 6.8 to 8.0 accelerated the inactivation 10-fold (not shown), suggesting that the process is favored upon formation of the thiolate-anion of DTT.

To investigate how the structure of QC is affected by disulfide bond cleavage, fluorescence spectra of the native, reduced, and unfolded protein were recorded. Usually, upon denaturation the emission maximum of proteins exhibits a tryptophan-mediated shift from a shorter wavelength to about 350 nm which corresponds to the fluorescence maximum of tryptophan in aqueous solutions (31). The native QC exhibited its fluorescence maximum at 340 nm (Figure 9A). Upon complete unfolding of the protein in 6 M GdmCl, the fluorescence intensity decreased and the emission maximum shifted to 355 nm, indicating a more hydrophilic environment of the tryptophan residues compared to the folded state. Interestingly, reduction of the disulfide with 15 mM DTT at pH 6.8 decreased fluorescence intensity only slightly, and a change in the fluorescence maximum did not occur (Figure 9B). No change in fluorescence intensity was detected in the absence of DTT (not shown). The differences in the fluorescence spectra of the native, reduced, and
denatured QC indicate a small conformational change of the protein possibly caused by the reduction of the disulfide bond.

**DISCUSSION**

Many human proteins cannot be purified from natural sources in amounts necessary for functional analyses. Heterologous expression is often the only choice to get sufficient amounts of the protein of interest. Among the various expression hosts, the methylotrophic yeast *P. pastoris* has been used successfully for many human proteins (29). In the current study, we demonstrate functional expression of human QC in *P. pastoris*. During expression, the protein was directed to the secretory pathway by fusion to the a-leader of *S. cerevisiae* and purified from the culture supernatant by a two-step purification procedure. Although QC activity was readily detectable when the protein was expressed in shake flasks, the rate of expression was improved 40-fold by fermentation. The overall yield of expression, however, was accompanied by heterogeneities in the glycosylation pattern of QC during long-term fermentations. Because the different glycoforms could not be separated efficiently by lectin-affinity- and ion-exchange chromatography, a fermentation time was chosen in which the altered glycoforms appeared to be minimal (Figure 3A). For mammalian QCs glycans were first shown for the QC from porcine pituitary (9, 16). The contribution of post-translational modifications found (approximately 2–4 kDa) in the case of the bovine QC (16) corresponds to the extent of glycosylation when the human QC is expressed in yeast. This yield of glycosylation is also in agreement with the structure of N-linked oligosaccharides of an invertase expressed as a heterologous protein in *P. pastoris*, too. The recombinant invertase contains more than 85% oligosaccharides in the size range M_{an}8–14GlcNAc_{2}, thus comparable to high-mannose oligosaccharides synthesized by animal cells (19). In addition, hyperglycosylation that is frequently observed when heterologous proteins are expressed in *S. cerevisiae* is commonly not found with proteins expressed in *P. pastoris* (17, 29). Heterogeneities in glycosylation, however, have been previously reported for other proteins expressed in *P. pastoris*, for instance, interleukin-17 (32) and HIV-1 envelope protein (33). Here, the glycosylation pattern of QC shifted to deglycosylated forms at later stages of growth (Figure 2). Previously, for *S. cerevisiae* a deglycosidase activity was reported that increased in cells reaching the stationary phase (34). Possibly, the appearance of the glycoforms of QC could be due to a post-translational cleavage of the sugar moieties. The human QC expressed in *P. pastoris* was deglycosylated quantitatively by only very low amounts of endoglycosidase H_{i} (Figure 4). Thus, both glycosylation sites seem to be easily accessible for a putative deglycosidase, even in the native, folded structure of QC, and both are possibly exposed at the protein surface. Human and bovine QC contain putative glycosylation sites at positions 49 and at positions 296 and 183, respectively, and both proteins show an overall sequence identity of 86%. The glycosylation sites, however, are within less conserved regions, implying that the protein conformation around these sites are less important for the catalytic properties of the QC. This is strengthened by the fact that catalytic parameters were unaffected by deglycosylation of the enzyme.

The nearly identical kinetic parameters of bovine and recombinant human QC obtained with the peptide H-Gln-Tyr-Ala-OH also reflect the high degree of homology between the enzymes. To date, estimates of the Michaelis constant and the turnover number for a native mammalian QC can only be achieved for H-Gln-Tyr-Ala-OH from literature data. On the basis of a molecular weight of 38–40 kDa for the purified bovine pituitary protein, a k_{cat} value of 225–235 s^{-1} can be calculated for the conversion of H-Gln-Tyr-Ala-OH at 37 °C (16). The corresponding Michaelis constant of 132 μM was determined in an earlier study (8). Thus, the kinetic parameters determined using the recombinant human QC are in excellent agreement with these earlier results obtained applying the highly homologous QC from bovine pituitary. This suggests that the proteins have similar if not identical catalytic competence, despite of heterologous expression and the presence of an N-terminal affinity tag in the recombinant human QC.

Remarkable differences in enzymatic activity were found between human QC expressed in *E. coli* (10, 11, 30) and that expressed in yeast, as reported here. At least partially, these differences might be due to the glutathione S-transferase fused to the N-terminus of QC expressed in *E. coli* (30). In the current study, the major portion of the QC protein expressed in *E. coli*, however, was inactive, suggesting that the active structure of QC was not formed as also indicated by the absence of the disulfide bond. This could be an additional reason for the apparent reduction in QC activity reported previously. Whether inactive proteins were formed by improper folding or by a lack of disulfide formation was not investigated in detail. However, initial experiments to separate the active and inactive QC forms by ion exchange chromatography failed. Furthermore, enzymatic activity could not be restored by addition of oxidized and reduced glutathione, a method often used for refolding of proteins in order to facilitate the correct formation of the disulfide bonds (35). Additionally, QC activity could not be detected when QC cDNA was expressed with the single base exchange in codon 164, which led to a tryptophan residue instead of a cysteine in this position. Although these results could also be interpreted in terms of misfolding, the loss of the disulfide bond of QC could also be a reason for inactivation, as indicated by the treatment of QC with DTT. Reducing cytosolic conditions are known to hinder the formation of disulfides in proteins (36), and therefore, translocation of the QC into a less reducing environment seems to be important for efficient formation of the enzymatically active structure.

The fluorescence spectra obtained for native, reduced, and unfolded QC showed that reduction of the disulfide bond resulted in a relatively small change of the protein conformation, indicated by a reduced fluorescence intensity. The unchanged fluorescence emission maximum also pointed to minor conformational differences. The concomitant loss of the enzymatic activity, however, clearly indicate an important role of the disulfide bond for the stabilization of the active protein structure. Furthermore, the fluorescence maximum at 339 nm of native QC indicates that not all tryptophan residues are in a hydrophobic environment. In such cases, the fluorescence maximum can still shift further into the blue range, as shown for RNase T1 (31) or prolyl oligopeptidase (37).
The QCs from *C. papaya* and its mammalian counterparts seem to be very similar with respect to molecular weights, subunit composition, and catalytic properties (30, 38). However, there was no sequence homology found between the enzymes (12), and their folding pattern was assumed to be different (14). Similarities were found between the predicted structure of human QC and bacterial zinc-dependent aminopeptidases that contain an α/β-structure (30). In addition, two different prediction methods (39, 40) used to calculate the portions of secondary structure from the amino acid sequence yielded 43% and 52% of α-helix and 15% and 16% of β-sheet for the recombinant human QC. Thus, the calculated values from the CD spectroscopy data and the predicted values are in the same range. Similar characterization experiments performed with QC from papaya latex revealed that the protein adopts an all-beta structure (14). Although the β-sheet content cannot be calculated from CD spectra without uncertainties (31), helical contents are mostly well reflected. Thus, the mammalian QCs seem to contain a pronounced α-helical secondary structure, in stark contrast to papaya QC.

To our knowledge, this is the first mammalian QC expressed and purified from an eukaryotic host. Due to the post-translational modifications of QC taking place in *P. pastoris*, this expression system has proven to be more favorable than bacterial expression. This might have also implications for other disulfide containing proteins that are expressed heterologously in these organisms. The most important advantage is provided by the fact that the catalytic competence of the human QC expressed in yeast is identical to the highly homologous QC purified from bovine pituitary, providing evidence that the recombinant protein resembles native QC very well. Therefore, by this study detailed enzymological and structural studies of human QC can be initiated.

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REFERENCES


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Continuous Spectrometric Assays for Glutaminyl Cyclase Activity

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The enzymatic conversion of one chromogenic substrate, L-glutamine-p-nitroanilide, and two fluorogenic substrates, L-glutaminyl-2-naphthylamide and L-glutaminyl-4-methylcoumarinylamide, into their respective pyroglutamic acid derivatives by glutaminyl cyclase (QC) was estimated by introducing a new coupled assay using pyroglutamyl aminopeptidase as the auxiliary enzyme. For the purified papaya QC, the kinetic parameters were found to be in the range of those previously reported for other glutaminyl peptides, such as Gln-Gln, Gln-Ala, or Gln-tert-buty1 ester. The assay can be performed in the presence of ammonium up to a concentration of 50 mM. Increasing ionic strength, e.g., potassium chloride up to 300 mM, resulted in an increase in enzymatic activity of about 20%. This is the first report of a fast, continuous, and reliable determination of QC activity, even in the presence of ammonium ions, during the course of protein purification and enzymatic analysis. © 2002 Elsevier Science (USA)

Several bioactive peptides and proteins (e.g., TRH, IgG, GnRH) contain a pyroglutamate residue at their N-terminal position. This feature was assumed to result from a spontaneous cyclization reaction of the N-terminal glutamine residue. However, enzymatic conversion by glutaminyl cyclase (QC; EC 2.3.2.5) could be shown (1–3).

Up to now, papaya latex has been the only plant source, in which this enzyme is found to be abundant, whereas several mammalian tissues have been shown to express QC (4, 5).

Little is known about the biological role of QC. It has been suggested that QC is responsible for modification of storage proteins during seed germination (6), as well as in vivo modification of bioactive peptides such as glucagon, MCP-2, TRH, and GnRH (4, 7, 8). These findings support the idea that the QC-catalyzed reaction might be important for protection of the N-terminus of bioactive peptides against exopeptidases. Moreover, this enzymatically catalyzed N-terminal formation of a pyroglutamic acid residue could be important in developing the proper receptor binding conformation of such peptides.

Interestingly, a number of pyroglutamate peptides are formed in tissues, although no QC activity has been detected there thus far, indicating a need for more detailed investigations (9). As an initial step in elucidating the function of QC in plants or animals, expression studies for various tissues and enzyme purification for further characterization are essential, and both require a reliable assay. In previously applied methods, QC activity was determined either by analyzing the products formed using HPLC (3, 10) or radioimmunoassay (11) or by detecting the release of ammonia spectrophotometrically (12). In the latter assay, the QC-catalyzed cyclization of the N-terminal glutamine residue is quantified by coupling the reaction with the conversion of NADH/H+ to NAD+ by glutamate dehydrogenase. Avoidance of any ammonium traces is an essential prerequisite, making this assay difficult to handle in some enzyme purification steps. Although the aforementioned methods are sensitive, they are all

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2 Abbreviations used: Boc, tert-buty1oxycarbonyl; Gln-AMC, L-glutaminyl-4-methylcoumarinylamide; Gln-pNA, L-glutaminyl-2-naphthylamide; GnRH, gonadotropin-releasing hormone; MCP-2, monocyte chemotactic protein-2; QC, glutaminyl cyclase; pNA, p-nitroanilide; TBA, triethylamine; THF, tetrahydrofuran; TRH, thyrotropin-releasing hormone; IgG, immunoglobulin G.
discontinuous and therefore time consuming and laborious. As a result of these disadvantages, we developed new continuous assays which allow determination of QC activity during purification and characterization. In contrast to the assays mentioned above, these new methods use the glutaminyl derivatives of p-nitroaniline, 7-amino-4-methylcoumarin, and 2-naphthylamine as substrates. Once cyclized into the respective pyroglutamic acid derivatives by QC, they are subsequently cleaved by pyroglutamyl aminopeptidase. The resulting liberation of p-nitroaniline, 7-amino-4-methylcoumarin, or 2-naphthylamine allows the reliable and convenient determination of QC activity.

MATERIALS AND METHODS

Materials

Lyophilized papaya latex and S-methylmethane thiosulfonate were purchased from Fluka (Seelze, Germany). Pyroglutamic acid p-nitroanilide and molecular mass standards for SDS–PAGE were provided by Sigma (Delsenohenf, Germany). Gln-AMC and Gln-βNA were from Bachem (Bubendorf, Switzerland). SP-Sepharose Fast Flow and Butyl-Sepharose 4 Fast Flow were obtained from Pharmacia Biotech (Uppsala, Sweden). Boc-L-glutamine was supplied by Bachem (Heidelberg, Germany). Pyroglutamyl aminopeptidase from Bacillus amyloliquefaciens, recombinantly expressed in Escherichia coli, was purchased from Unzyme Laboratories (Hørsholm, Denmark) and Tris as well as Tricine from Serva (Heidelberg, Germany).

Synthesis of Gln-pNA

Boc-Gln-pNA. Boc-Gln-OH (2.46 g; 10 mmol) was dissolved in THF (20 ml) by adding 0.81 ml (10 mmol) pyridine and 1.39 ml (10 mmol) TEA and solvation was completed by a brief warming of the solution. After the mixture was cooled down to -10°C, 1.23 ml (10 mmol) pivaloyl chloride was added, and the clear solution was stirred for 10 min at 0°C. Upon completed formation of the mixed anhydride, 1.311 g (9.5 mmol) p-nitroaniline was added. Subsequently, the mixture was stirred for 1 h at 0°C and left overnight at room temperature. In the usual workup the solvent was removed, and the residue was partitioned between ethyl acetate and aqueous HCl (15% in water), followed by a subsequent washing with brine, saturated aqueous solution of KHCO₃, and brine again. The organic layer was separated and the solvent was removed. Chromatography on silica gel using a CHCl₃:MeOH (1:3, v/v) gradient resulted in a pure Boc-Gln-pNA as a faint yellow oil. The overall yield was 2.93 g (80%).

H-Gln-pNA·HCl. Boc-Gln-pNA was treated with 20 ml HCl solution (4 N in dioxane) until the starting material was no longer detectable. After solvent removal, the resulting material was recrystallized from methanol/ether. The yield was 2.17 g (90%), HPLC purity 99.35% (water/ACN/TFA). ¹³C NMR (CD₃OD) δ 24.95 (CH₃), 30.11 (CH₂), 53.27 (CH—NH₂), 121.55 (CH—phenyl), 122.4 (CH—phenyl), 143.87 (═C—phenyl), 147.23 (═C—NO₂), 161.65 (CONH), 173.88 (CONH).

Purification of QC from Papaya Latex

QC from papaya latex was prepared using the BioCAD 700E (Perceptive Biosystems, Wiesbaden, Germany) with a modified version of a previously reported method (13). Fifty grams of latex was dissolved in water and centrifuged as described (13). Inactivation of proteases was performed with S-methylmethane thiosulfonate, and the resultant crude extract was dialyzed (13, 23).

SP-Sepharose Fast Flow. After dialysis, the entire supernatant was loaded onto a (21 × 2.5-cm i.d.) SP-Sepharose Fast Flow column, equilibrated with 100 mM sodium acetate buffer, pH 5.0 (flow rate 3 ml/min). Elution was performed in three steps by increasing the sodium acetate buffer concentration at a flow rate of 2 ml/min. The first step was a linear gradient from 0.1 to 0.5 M acetate buffer in 0.5 column volume. The second step was a linear increase in buffer concentration from 0.5 to 0.68 M in 4 column volumes. During the last elution step, 1 column volume of 0.85 M buffer was applied. Fractions (6 ml) containing the highest enzymatic activity were pooled. Concentration and buffer changes to 0.02 M Tris/HCl, pH 8.0, were performed via ultrafiltration (Amicon; molecular mass cut-off of the membrane 10 kDa).

Butyl-Sepharose 4 Fast Flow. Ammonium sulfate was added to the concentrated papaya enzyme, obtained from the ion-exchange chromatography step to a final concentration of 2 M. This solution was applied onto a (21 × 2.5-cm i.d.) Butyl-Sepharose 4 Fast Flow column (flow rate 1.3 ml/min), equilibrated with 2 M ammonium sulfate, 0.02 M Tris/HCl, pH 8.0. Elution was performed in three steps with decreasing concentrations of ammonium sulfate. During the first step a linear gradient from 2 to 0.6 M ammonium sulfate, 0.02 M Tris/HCl, pH 8.0, was applied for 0.5 column volume at a flow rate of 1.3 ml/min. The second step was a linear gradient from 0.6 to 0 M ammonium sulfate, 0.02 M Tris/HCl, pH 8.0, in 5 column volumes at a flow rate of 1.5 ml/min. The last elution step was carried out by applying 0.02 M Tris/HCl at pH 8.0 for 2 column volumes at a flow rate of 1.5 ml/min. All fractions containing QC activity were pooled and concentrated by ultrafiltration. The resultant homogeneous QC was stored at -70°C. Final protein concentrations were determined using the method of Bradford (14),
compared to a standard curve obtained with bovine serum albumin.

Assays

All measurements were performed with the BioAssay Reader HTS 7000Plus for microplates (Perkin–Elmer) at 30°C.

Spectrophotometric assay for QC. The assay consisted of the chromogenic substrate Gln-pNA (1 mM), pyroglutamyl aminopeptidase (0.25 U), and an appropriate amount of QC in a final volume of 0.25 ml buffer (0.05 M Tricine/NaOH, pH 8.0). This pH was reported to be within the optimal range for the catalysis of both QC and pyroglutamyl aminopeptidase (9, 15). Due to the composition of the storage buffer of pyroglutamyl aminopeptidase, assay mixtures contained cysteamine (0.1 mM), sodium chloride (2 mM), EDTA (0.1 mM), and 2% (v/v) glycerol.

Reactions were initiated by addition of QC and preincubated for 2 min, and subsequently absorption was monitored at 405 nm for 8–15 min. QC activity was calculated using an absorption coefficient of 6710 L/mol, which was determined from a standard curve of p-nitroaniline under assay conditions.

Fluorometric assays for QC. Glutaminyl cyclase activity was evaluated fluorometrically using either Gln-AMC or Gln-bNA. The samples consisted of the fluorogenic substrate (0.05 mM), 0.25 U pyroglutamyl aminopeptidase in 20 mM Tris/HCl, pH 8.0, containing 200 mM potassium chloride, and an appropriately diluted aliquot of papaya glutaminyl cyclase. Excitation/ emission wavelengths were 380/465 nm in the case of Gln-AMC and 320/410 nm in the case of Gln-bNA. The assay reactions were initiated by addition of glutaminyl cyclase. Monitoring of progress curves was started immediately after initiation. QC activity was determined from standard curves of 7-amino-4-methylcoumarin and β-naphthylamine under assay conditions.

Assay for pyroglutamyl aminopeptidase. Measurements of pyroglutamyl aminopeptidase activity were carried out using pGlu-pNA as substrate. In order to maintain conditions identical to those used in the QC assay, samples (250 μl) contained 0.1 mM cysteamine, 2 mM sodium chloride, 0.1 mM EDTA, 2 mM pGlu-pNA (stock solution in dimethyl sulfoxide), 2% (v/v) glycerol, and an diluted aliquot of pyroglutamyl aminopeptidase in 0.05 M Tricine/NaOH, pH 8.0. The final concentration of 1% (v/v) dimethyl sulfoxide did not interfere with the activity of pyroglutamyl aminopeptidase. One unit is defined as the amount of enzyme that hydrolyzes 1 μmol pGlu-pNA per minute under the conditions described. The activity was expressed in units using an absorption coefficient of 6690 L/mol, which was obtained from a standard curve. The specific activity of the enzyme preparation was approximately 4 units/mg. The pyroglutamyl aminopeptidase was stored in a solution consisting of Tris/HCl (6 mM), cysteamine (2 mM), sodium chloride (40 mM), EDTA (2 mM), 50% glycerol, pH 8.0. The enzyme was stable for several months at −20°C.

RESULTS AND DISCUSSION

Spectrophotometric Assay

The spectrophotometric assay is based on the detection of p-nitroaniline at 405 nm, one of the products generated in the coupled assay. The first reaction is the conversion of Gln-pNA into pGlu-pNA, catalyzed by QC. pGlu-pNA, in turn, is hydrolyzed in the second reaction by the abundant pyroglutamyl aminopeptidase, which leads to the terminal products pyrogglutamate and pNA. Thus, QC is the enzyme being analyzed, and pyroglutamyl aminopeptidase represents the auxiliary enzyme. p-Nitroaniline is released in equimolar amounts to the Gln-pNA converted by QC. Hence, QC activity is directly related to the amount of p-nitroaniline released and can therefore easily be quantified. For estimation of the conversion rate from Gln-pNA to pGlu-pNA, this first step has to be rate determining for the complete assay. Previous theoretical investigations on the kinetics of irreversible coupled enzyme assays showed the possibility of calculating the required amount of an auxiliary enzyme for the development of a reliable method (16). The calculation was performed according to Eq. [1].

\[ V_2 = \frac{K_{\text{m2}} \times \ln(1 - [\text{pGlu-pNA}]_t/[\text{pGlu-pNA}]_{\text{SS}})}{t}, \]

where \( K_{\text{m2}} \) is the Michaelis constant and \( V_2 \) the maximal velocity of the reaction catalyzed by the auxiliary enzyme, pyroglutamyl aminopeptidase. \([\text{pGlu-pNA}]_t\) represents the concentration of pGlu-pNA at time \( t \) after initiation of the reaction. \([\text{pGlu-pNA}]_{\text{SS}}\) describes the steady-state concentration of pGlu-pNA under these conditions. Equation [1] is valid if the initial substrate concentration does not change significantly during the considered time and the second reaction follows a first-order rate law ([pGlu-pNA] \( \leq K_{\text{m2}} \)). Using the known \( K_{\text{m2}} \), the required amount of auxiliary enzyme can be calculated from \( V_2 \). We determined a \( K_{\text{m2}} \) value of 710 ± 50 μM for pyroglutamyl aminopeptidase and pGlu-pNA under the assay conditions, which corresponds to data obtained from the literature (17). Using this value and assuming that the definition of 1 unit of auxiliary enzyme refers to saturated substrate concentrations, we estimated 0.25 U pyroglutamyl aminopeptidase to be required in the sample volume to reach 95% of the steady-state concentration of pGlu-pNA after 130 s. Thus, linear progress curves
were expected after a lag time of 2 min, independent of the concentration of Gln-pNA.

As shown in Fig. 1, Gln-pNA is recognized as a substrate for QC, demonstrated by an increase in absorption at 405 nm. There was no increase in absorption without QC, indicating that formation of p-nitroaniline is dependent on the presence of QC. Linear product formation was observed at 0.25 U pyroglutamyl aminopeptidase and a preincubation time of 120 s, verifying the reliability of the assay and the calculation made above regarding the excess of pyroglutamyl aminopeptidase required. To counteract rapid substrate consumption during preincubation, the concentration of QC was adjusted precisely by dilution. Based on this, the assay was carried out at an activity concentration of 1 unit/ml pyroglutamyl aminopeptidase, giving reproducible progress curves of glutaminyl cyclase activity under the conditions used. Increasing the amount of pyroglutamyl aminopeptidase shortened the lag time, but had no influence on the slope of the progress curves.

In addition, there was a linear relationship between the initial velocity and the concentration of QC (Fig. 2). This confirms that the rate of catalysis is dependent on the QC-mediated conversion of Gln-pNA to pGlu-pNA.

The feasibility of the novel assay was tested during the QC purification from papaya latex, demonstrated here by determination of QC activity in fractions of the first purification step (Fig. 3). Highly purified QC was generated by only two separation steps. A rapid purification was reached by implementing a multilevel gradient in the initial ion-exchange chromatography followed by a hydrophobic interaction chromatography. Similar to the purification procedure described by Zerhouni et al. (13), QC was eluted among the last enzymes that can be purified from papaya by cation-exchange chromatography. This is somewhat surprising since the proteins should be eluted in the order of their increasing isoelectric points (pI). However, the QC of our preparation showed a pI of 9.4–9.6, determined by isoelectric focusing in agarose gels (data not shown). This value is more alkaline than the reported isoelectric point of papain (pI 7.5), but noticeably more acidic than that of papaya proteinase A (pI 11) (18), the most basic of the papaya proteinases (19). The reasons for the anomalous order of elution were not examined in detail, but the phenomenon might be explained by assuming that highly basic regions on the surface of QC may account for strong electrostatic interactions with the cation exchange resin. The high hydrophobicity reported previously (13) might be attributed to other regions, established in the correctly folded protein. Although the overall yield of the purification presented here was approximately 25%, three times less than that previously published (13), our strategy was much less time consuming. Hence, the reduced overall yield of the purification can be easily compensated by the yield of QC in the latex of C. papaya. Thirty-five milligrams of homogeneous QC was obtained from
150 g of lyophilized papaya latex using the new procedure.

Although Gln-pNA is a dipeptide surrogate, the kinetic parameters for its conversion by QC correspond to those of other dipeptides. Using the continuous spectrophotometric assay presented here, a Michaelis constant of 102 ± 4 μM was determined (Fig. 4). This corresponds to 90 ± 20 μM detected for Gln-Gln and 210 ± 40 μM for Gln-Ala under comparable conditions (6). Based on the assumption of a monomeric protein with a molecular mass of 33 kDa, determined by gel electrophoresis (13), a first-order rate constant for breakdown of the enzyme/substrate complex to the products (k_cat) of 28 ± 1 s⁻¹ at 30°C was determined. This corresponds to the value of 50 s⁻¹ at 37°C reported for Gln-tert-butyl ester (13).

In order to verify these parameters, they were evaluated by an alternative method (12). In this assay, detection of QC activity was accomplished by conversion of the ammonia formed into glutamate, catalyzed by glutamate dehydrogenase in the presence of α-ketoglutaric acid and NADH/H⁺. In the subsequent reaction, consumption of NADH/H⁺ can be monitored spectrophotometrically at 340 nm. Due to the overlapping absorption of the substrate Gln-pNA at 340 nm, a detection wavelength of 355 nm was found to be optimal when using this substrate. Kinetic parameters computed for the conversion of Gln-pNA correlate well with those obtained using the novel assay described above, e.g., K_m and k_cat were calculated to be 99 ± 6 μM and 27 ± 1 s⁻¹, respectively, thus indicating the validity of the continuous method.

In order to substantiate the feasibility of the new assay in more detail, the effect of ammonia concentration on the conversion rate of Gln-pNA to pGlu-pNA was recorded at constant ionic strength (μ = 0.07 M). No change in activity could be observed in the range of ammonia concentration analyzed, indicating that the assay can be performed up to 50 mM ammonia. This suggests that QC lacks product inhibition by ammonia. Obviously, the release of ammonia is not the rate-limiting step in the catalysis by papaya QC.

Ionic strength was tested as another important parameter in establishing the new QC assay in enzyme characterization. As a prerequisite, constant activity of the auxiliary enzyme is necessary under the chosen conditions, e.g., time to reach steady-state conditions (Eq. [1]) is unaltered. This prerequisite was fulfilled, since activity of pyroglutamyl aminopeptidase was enhanced by increasing ionic strength (data not shown). As demonstrated in Fig. 5, activity of QC increased steadily up to 300 mM KCl and was almost constant up to 500 mM KCl.
to 500 mM. The overall activation by increasing ionic strength was approximately 20%. A very similar dependence was observed for NaCl (not shown). Thus, within the QC assay a constant ionic strength is important to avoid activating effects of abundant ions. The effect of ionic strength on QC activity might be caused by (i) weakened salt bridges, leading to structural changes and higher enzymatic activity, and by (ii) altered dissociation constant of catalytic groups of the enzyme. Such altered constants at increasing ionic strength were reported previously for lysozyme (20).

Fluorometric Assays

Based on the results obtained with the spectrophotometric assay, two further assays using fluorometric detection were developed. For convenience, the commercially available fluorogenic substrates Gln-βNA and Gln-AMC were purchased and tested as substrates of papaya glutaminyl cyclase. As expected from data in the literature, pyroglutamyl-βNA and pyroglutamyl-AMC are potent substrates for the auxiliary enzyme (21, 22). In agreement with these reports, we determined $V_{max}/K_m$ values for the fluorogenic substrates that were approximately 20-fold higher than that of pGlu-pNA. This potency of the auxiliary enzyme for hydrolysis of the potential intermediates emerging in the QC assay results in drastically reduced lag times when using the same concentration (1 U/ml) of pyroglutamyl aminopeptidase compared to the spectrophotometric assay based on release of pNA. The times required to reach steady-state conditions were calculated according to the method mentioned above and were found to be less than 5 s for Gln-AMC as well as Gln-βNA. Thus, steady-state conditions were reached within the time required for mixing and beginning of data monitoring. In fact, linear progress curves were obtained in the QC assay for the conversion of both fluorogenic substrates, immediately after initiation of the reaction (Fig. 6A). In the case of both substrates, the observed change in fluorescence was found to increase linearly during the time of incubation. There was no increase in fluorescence detected in the absence of QC or pyroglutamyl aminopeptidase.

As shown in Fig. 6B, linearity was observed between the reaction rate and the enzyme concentration when 0.05 mM substrate was used under these conditions, indicating that the compounds can be used as sensitive substrates for glutaminyl cyclase. Furthermore, in comparison to the chromogenic substrate Gln-pNA, the assays described here seem to be approximately 1000 times more sensitive and are capable of detecting enzyme concentrations as low as 0.4 ng/ml.

Kinetic analyses of the enzymatic conversion of both substrates revealed that the enzyme reactions fit Lineweaver–Burk plots (data not shown). The $K_m$ values for conversion of the substrates were 43 ± 4 µM for Gln-βNA and 52 ± 5 µM in the case of Gln-AMC. Interestingly, these are the lowest values that were determined for payaya glutaminyl cyclase, and they are noticeably less than that of the chromogenic substrate Gln-βNA. The values of $k_{cat}$ for conversion of Gln-βNA (46 s⁻¹) and Gln-AMC (31 s⁻¹) by QC are similar to that of Gln-pNA. As a consequence, it can be concluded that the chromogenic and fluorogenic substrates described here can be regarded as the best for papaya QC with respect to their $k_{cat}/K_m$ values. Both fluorogenic substrates seemed to show weak substrate inhibition at concentrations higher than 10 $K_m$. However, at the concentrations used, this inhibition was minimal and did not interfere with the determination of the kinetic parameters.

A limitation of feasibility, also in the case of other assays of QC, is the susceptibility of the chromogenic and fluorogenic substrates to aminopeptidase attack. Thus, in some applications, addition of aminopeptidase inhibitors will be necessary. Accordingly, we tested three peptidase inhibitors, i.e., bestatin (100 µg/ml), aprotinin (50 µg/ml), and EDTA (5 mM), concerning their influence on the assay, using Gln-βNA as substrate. None of the three substances interfered with the assay, making them useful as additives to inhibit peptidases in crude samples.

Finally, the substrates were also tested upon conversion by human glutaminyl cyclase, recombinantly expressed in the yeast Pichia pastoris (published elsewhere). All three assays also worked with this enzyme.
(data not shown), making the procedures applicable for plant and animal sources of QC.

To our knowledge, these are the first continuous assays described for QC that can be implemented during protein purification. Among the advantages of the presented methods, the continuous measurement and less time consumption make the methods more favorable compared to previously developed methods.

The most important advantage of the new methods, in comparison to a previously described assay (12), is the fact that these methods can be performed in the presence of ammonia. Thus, various protein purification steps using ammonium sulfate can be easily monitored.

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REFERENCES


Glutaminyl cyclases (QC) catalyze the intramolecular cyclization of N-terminal glutamine residues of peptides and proteins. For a comparison of the substrate specificity of human and papaya QC enzymes, a novel continuous assay was established by adapting an existing discontinuous method. Specificity constants ($k_{cat}/K_m$) of dipeptides and dipeptide surrogates were higher for plant QC, whereas the selectivity for oligopeptides was similar for both enzymes. However, only the specificity constants of mammalian QC were dependent on size and composition of the substrates. Specificity constants of both enzymes were equally pH-dependent in the acidic pH-region, revealing a $pK_a$ value identical to the $pK_a$ of the substrate, suggesting similarities in the substrate conversion mode. Accordingly, both QCs converted the L-$\beta$homoglutaminyl residue in the peptide H-$\beta$homoGln-Phe-Lys-Arg-Leu-Ala-NH$_2$ and the glutaminyl residues of the branched peptide H-Gln-Lys(Gln)-Arg-Leu-Ala-NH$_2$ as well as the partially cyclized peptide H-Gln-cyclo(Ne-Lys-Arg-Pro-Ala-Gly-Phe). In contrast, only QC from C. papaya was able to cyclize a methylated glutamine residue, while this compound did not even inhibit human QC-catalysis, suggesting distinct substrate recognition pattern. The conversion of the potential physiological substrates [Gln$^1$]-gastrin, [Gln$^1$]-neurotensin and [Gln$^1$]-fertilization promoting peptide indicates that human QC may play a key role in posttranslational modification of most if not all pGlu-containing hormones.

**Key words:** Glutamine cyclotransferase/$\beta$Homoglutamine/5-Oxo-L-proline/Pyroglutamic acid.

**Introduction**

Glutaminyl cyclases (QC, EC 2.3.2.5) catalyze the intramolecular cyclization of N-terminal glutamine residues into pyroglutamic acid (pGlu) with liberation of ammonia (Figure 1). In 1963, the first QC was isolated from the latex of the tropical plant Carica papaya (Messer, 1963). Twenty four years later, a corresponding enzymatic activity was discovered in mammalian pituitary homogenates (Busby et al., 1987; Fischer and Spiess, 1987). This enzyme is thought to be responsible for the generation of the N-termini of peptide hormones and proteins containing pGlu. The conversion of Gin into pGlu by a QC was demonstrated for the precursors of thyroid hormone-releasing hormone (TRH) and gonadotropin-releasing hormone (GnRH) (Busby et al., 1987; Fischer and Spiess, 1987). Experiments revealed a colocalization of the QC with its putative catalytic products in bovine pituitary, thereby supporting a potential function in peptide hormone synthesis. The physiological function of plant QC is unknown. In case of QC from C. papaya, a role in plant defense reactions was suggested (El Moussaoui et al., 2001). Putative QCs from other plants have been identified by sequence comparisons (Dahl et al., 2000), but physiological functions of these enzymes are yet to be characterized.

An initial comparison of plant and animal QCs reveals several similarities. QCs from both sources are monomeric with similar molecular masses of 33 – 40 kDa (Pohl et al., 1991; Zerhouni et al., 1998). They are glycoproteins synthesized via the secretory pathway, and the carbohydrates contribute approximately 2 – 4 kDa to their molecular mass (Pohl et al., 1991; Dahl et al., 2000). Furthermore, all QCs show a strict specificity for L-glutamine at the N-terminal position of the substrates and their kinetic behavior was found to obey the Michaelis-Menten equation (Consalvo et al., 1988; Pohl et al., 1991; Gololobov et al., 1996). Interestingly, the primary structures of QC from C. papaya and that of the highly conserved QC from mammals did not reveal any sequence homology (Dahl et al., 2000). In addition, plant QC was described to contain extensive $\beta$-sheet structure (Oberg et al., 1998), in contrast to a recent CD-spectral structure determination.
of the human enzyme (Schilling et al., 2002a). The structure of the mammalian QCs is remarkably homologous to the bacterial aminopeptidases (Bateman et al., 2001), whereas the plant QCs appear to belong to a separate enzyme family (Dahl et al., 2000). This led to the conclusion that QCs from plants and animals have different evolutionary origins.

Due to the apparent divergence, different properties and consequently different functions are to be expected when comparing QC isoforms from plants and animals. To address this question, we performed a direct comparison of plant and animal QC by analysis of the QC from C. papaya and the recombinant human QC, including a detailed analysis of catalytic properties. Based on the previously used discontinuous assay methods, only limited information concerning substrate specificity and QC selectivity is currently known. So far, kinetic parameters have only been determined for amino acid derivatives or di- and tripeptides (Bateman, 1989; Song et al., 1994; Gololobov et al., 1996; Sykes et al., 1999). Gonadotropin-releasing hormone and thyrotropin releasing-hormone have been investigated using HPLC as the only larger potential substrates of QC (Fischer and Spiess, 1987).

Here, we report the first comprehensive characterization of longer peptides as QC substrates using a novel continuous spectrophotometric assay. This method enabled a detailed investigation of the impact of N-terminal glutamate/glutamine modifications, of the nature of the C-terminal amino acids and the length of the substrates on QC-catalysis. Furthermore, kinetic parameters of QC-catalysis of potential physiological substrates were also determined for the first time.

Results

Spectrophotometric Assay

The discontinuous assay introduced by Bateman (1989) was modified to a continuous method, applicable for microplates. In the assay, glutamic dehydrogenase and its substrates α-ketoglutaric acid and NADH/H⁺ are coupled to QC activity by liberation of ammonia from the glutamine residue. Accordingly, QC activity is reflected by a decrease in absorbance at 340 nm caused by the ammonia release and subsequent consumption of NADH/H⁺ due to formation of glutamate from α-ketoglutaric acid. The amount of the auxiliary enzyme necessary to obtain a sufficiently short ‘lag time’, i.e. the time in which a steady-state is reached between the reaction catalyzed by the investigated enzyme and the reaction catalyzed by the auxiliary enzyme, was first calculated using a previously described method (McClure, 1969), an approach that was already used to develop fluorescence-based continuous assays for QC (Schilling et al., 2002b). Under saturating concentrations of NADH/H⁺ and α-ketoglutaric acid (McClure, 1969), 30 U/ml glutamic dehydrogenase was calculated to be a sufficient excess to reach 95% of the steady-state concentration of ammonia after 20 s, i.e. a time required for starting the reaction and mixing of the assay constituents. Thus, linear progress curves should be observed upon starting data acquisition. Indeed, linear progress curves were detected under these conditions (Figure 2). A linear relationship was observed between the activity and the concentration of QC. The kinetic parameters obtained for H-Gln-Gln-OH in the continuous assay (Table 1) were in good agreement with those obtained with the discontinuous method (Km = 175±18 μM, kcat = 21.3±0.6 s⁻¹). In addition, the kinetic parameters for conversion of the substrates H-Gln-Ala-OH and H-Gln-NH₂ by the papaya QC (Table 1) correspond well to those determined with a direct method at pH 8.8 and 37°C (Gololobov et al., 1996).

pH Dependence

The pH dependence of catalysis of human and papaya QC was investigated under first-order rate law conditions, thus reflecting the impact of pH on the specificity constant kcat/Km. For this purpose, pyroglutamyl aminopeptidase was used as auxiliary enzyme with Gln-βNA as substrate in the coupled assay. Pyroglutamyl aminopeptidase was shown to be active and stable between pH 5.5 – 8.5 (Tsuru et al., 1978), hence, this assay allowed QC analysis in this pH-range. The rate profiles obtained fit to classical bell-shaped curves (Figure 3). For human QC a very narrow pH dependence with an optimum at about pH 7.8 – 8.0 was observed (Figure 3A), and the rate...
decreased at more basic pH. Also papaya QC exhibited optimal activity near pH 8.0. However, no drop of $k_{cat}/K_m$ occurred up to a pH of 8.5 (Figure 3B). Evaluation of rate profiles revealed that at 23°C, pK$_a$ values were 7.17±0.02 and 7.15±0.02 for human QC and papaya QC, respectively. These pK$_a$ values are identical to the pK$_a$ of the substrate H-Gln-βNA (7.16±0.01). The respective pK$_a$ values obtained by titration and kinetic analysis of the pH dependence shifted toward 6.97±0.01 and 7.03±0.02 at 30°C. Thus, substrate binding and/or conversion by both QC forms seems to be dependent on an unprotonated α-amino group.

The shape of the pH-profile of human QC in the basic pH-range was obviously due to dissociation of a group with a pK$_a$ of approximately 8.5. For papaya QC, the limited stability of the auxiliary enzyme resulted in collection of insufficient data under basic pH conditions. Consequently, a reliable determination of a potential second pK$_a$ value could not be achieved. This is also reflected by the fact that fitting the data to a single dissociation model gave a very similar result (pK$_a$ 7.13±0.03) compared to a double dissociation model, indicating that both pK$_a$ values are fairly separated.

### pH Stability

The stability of the QCs was analyzed by preincubating both enzymes at 30°C for 30 min at different pH values between pH 4 and pH 10, followed by activity tests under standard conditions (Figure 4). The QC from papaya latex was found to be stable in the pH-range studied (Zerhouri et al., 1998). In contrast, the human QC was stable only between pH 7 and 8.5, but exhibited a remarkable instability above pH 8.5 and below pH 6. Thus, pH 8 seemed to be optimal for activity and stability of plant and human QC. Consequently, this pH value was used to compare the substrate specificity of both QCs.
Substrate Specificity

Di-, Tripeptides and Dipeptide Surrogates The substrate specificity of both QCs was analyzed with 30 potential substrates (Table 1). Nearly all of the short peptides were more efficiently converted by the papaya QC compared to the human QC. This is particularly obvious in case of the conversion of L-glutamine. Whereas the plant enzyme was able to react with L-glutamine, no reactivity of human QC was detected. Both enzymes were highly active toward substrates carrying large hydrophobic residues in the penultimate position of peptides, such as H-Gln-Tyr-Ala-OH, H-Gln-Phe-Ala-NH₂, or H-Gln-Trp-Ala-NH₂, or H-Gln-AMC and H-Gln-βNA, as compared to other tripeptides or dipeptide substrates. Plant as well as human QC were remarkably inhibited by H-Gln-βNA at high concentrations, revealing almost identical $K_i$ values of about 1.2 mM (Table 1). Minor substrate inhibition was detected for H-Gln-AMC. Hence, low solubility of the substance hampered determination of the $K_i$ value. A striking difference in specificity of the plant and the animal QC was observed for H-Gln-OtBu. Whereas the ester was converted by the papaya QC with similar specificity compared to dipeptide substrates, its turnover was more than one order of magnitude slower by human QC.

Oligopeptides In addition to several dipeptides and tripeptides, a number of putative oligopeptide substrates was tested with papaya and human QC (Table 1). Interestingly, the overall difference in the specificities between human and plant QC was not as large for tetrapeptides as was observed for dipeptide and tripeptide substrates. This indicates that the amino acids in the 3rd and 4th position still affect the kinetic behavior especially of human QC, leading to similar specificity constants. However, H-Gln-Pro-Tyr-Phe-NH₂, a tetrapeptide with proline in the penultimate position, yielded noticeably reduced $k_{cat}/K_m$ values, providing an exception (Table 1). The reduction in the specificity was more pronounced for human QC, leading to an approximately 8-fold difference in the $k_{cat}/K_m$ value as compared to papaya QC. Slightly reduced specificity constants of human QC were also observed for the conversion of substrates with a positively charged amino acid on the C-terminal side of glutamine, such as H-Gln-Arg-Tyr-Phe-NH₂, H-Gln-Arg-Gly-Ile-NH₂ and H-Gln-Lys-Arg-Leu-NH₂, as compared to other tetrapeptides. Apparently, the reduced specificity was mainly due to a smaller turnover number. This effect was not observed for the plant enzyme.

In contrast to the selectivity of papaya QC for dipeptides, human QC was more selective for some tetrapeptides. The highest selectivity of human QC was recorded for peptides containing bulky hydrophobic residues in the 3rd and 4th amino acid position, which indicate hydrophobic interactions with the enzyme. Comparing the kinetic parameters for the respective peptides, the altered specificity seems to be mainly due to lower $K_m$ values, since the turnover numbers for conversion of the

Fig. 3 pH Dependence of Human (A) and Papaya (B) QC. Determinations were carried out under first-order rate conditions using Gln-βNA as substrate. In case of human QC, a buffer system providing a constant ionic strength according to Ellis and Morrison (1982) was used, consisting of 25 mM MES, 25 mM acetic acid and 50 mM Tris. Due to a slightly inhibiting effect of Tris, papaya QC was investigated using a 50 mM MOPS buffer. The ionic strength was adjusted to 0.05 M by addition of NaCl. The rate profiles were evaluated by fitting the data according to equations that account for two dissociating groups. In case of papaya QC, a $pK_a$ value of 7.13 ± 0.03 was obtained by fitting of the data according to a single dissociation model.

Fig. 4 Effect of pH on the Stability of the QC from Papaya Latex and Human QC. An enzyme stock solution was diluted 20-fold in 0.1 mM buffer of various pH values (pH 4 – 7, sodium citrate; pH 7 – 10, sodium phosphate). Enzyme solutions were incubated at 30°C for 30 min, and subsequently enzymatic activity was analyzed according to the standard protocol.
peptides were found to be similar. Obviously, the higher selectivity of human QC is due to stronger binding of the more hydrophobic substrates to the enzyme.

An increasing \( k_{\text{cat}}/K_m \)-ratio was also found for peptides of varying length consisting of the N-terminal glutamine residue and alanine residues as substrates of human QC (Table 2). While human QC was more selective for substrates of a length up to a pentapeptide, there was no such a trend with the papaya QC. Human QC was less active toward a serine-containing peptide, indicating that also the nature of the substrate side chains of the amino acids close to the reaction center is of importance.

### Influence of Ionic Strength on Catalysis

To analyze the influence of ionic strength on the substrate specificity, the kinetic parameters for cyclization of several substrates were determined in presence and absence of 0.5 M KCl (Table 3). Interestingly, the specificity of both QCs for substrates with uncharged backbone did not change significantly by salt addition. For substrates such as H-Gln-Ala-OH and H-Gln-Glu-OH, however, addition of KCl decreased specificity in case of the human enzyme. As indicated by the kinetic parameters, this effect was due to a higher \( K_m \) and a slightly reduced \( k_{\text{cat}} \) value. The papaya QC did not show altered kinetic parameters upon salt addition. The effect did not seem to be due to the negatively charged substrate as such, since similar kinetic parameters were found for the negatively charged peptide H-Gln-Glu-Asp-Leu-NH₂. With the positively charged substrates H-Gln-Arg-Gly-Ile-NH₂ and H-Gln-Lys-Arg-Leu-NH₂ addition of salt revealed a positive effect on catalysis for both QCs, as indicated by a lower \( K_m \) value and a slightly higher turnover number.

### Physiological Substrates

Physiological substrates of papaya QC are unknown. For human QC the compounds

Table 2  Dependence of the Kinetic Parameters on the Substrate Size.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Human QC</th>
<th>Papaya QC</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_m ) (µM)</td>
<td>( k_{\text{cat}} ) (s⁻¹)</td>
<td>( k_{\text{cat}}/K_m ) (µM⁻¹ s⁻¹)</td>
</tr>
<tr>
<td>H-Gln-Ala-NH₂</td>
<td>155±9</td>
<td>40.1±0.9</td>
</tr>
<tr>
<td>H-Gln-Ala-Ala-NH₂</td>
<td>87±3</td>
<td>76.3±0.7</td>
</tr>
<tr>
<td>H-Gln-Ala-Ala-Ala-NH₂</td>
<td>65±3</td>
<td>60.5±0.7</td>
</tr>
<tr>
<td>H-Gln-Ala-Ser-Ala-Ala-NH₂</td>
<td>79±6</td>
<td>55.3±1.6</td>
</tr>
</tbody>
</table>

Reactions were performed under identical conditions as described in Table 1.

Table 3  Dependence of the Catalytic Parameters \( k_{\text{cat}} \) and \( K_m \) on the Ionic Strength for Substrates of Varying Length and Charge.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Papaya QC 0.05 M Tricine-NaOH, pH 8.0</th>
<th>Human QC 0.05 M Tris-HCl, pH 8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_m ) (µM)</td>
<td>( k_{\text{cat}} ) (s⁻¹)</td>
<td>( k_{\text{cat}}/K_m ) (µM⁻¹ s⁻¹)</td>
</tr>
<tr>
<td>H-Gln-NH₂</td>
<td>434±15</td>
<td>43.4±0.4</td>
</tr>
<tr>
<td>H-Gln-JNA</td>
<td>36±2</td>
<td>48.8±1.0</td>
</tr>
<tr>
<td>H-Gln-Ala-OH</td>
<td>137±7</td>
<td>69.7±9.9</td>
</tr>
<tr>
<td>H-Gln-Glu-OH</td>
<td>98±5</td>
<td>45.0±0.5</td>
</tr>
<tr>
<td>H-Gln-Trp-Ala-NH₂</td>
<td>79±5</td>
<td>138±3.3</td>
</tr>
<tr>
<td>H-Gln-Ang-Gly-Ile-NH₂</td>
<td>106±8</td>
<td>52.9±1.2</td>
</tr>
<tr>
<td>H-Gln-Lys-Ang-Leu-NH₂</td>
<td>102±7</td>
<td>50.1±3</td>
</tr>
<tr>
<td>H-Gln-Glu-Asp-Leu-NH₂</td>
<td>109±5</td>
<td>52.4±0.7</td>
</tr>
</tbody>
</table>

Reactions were carried out at 30°C. *Substrate inhibition.

peptide inhibitors. Obviously, the higher selectivity of human QC is due to stronger binding of the more hydrophobic substrates to the enzyme.

An increasing \( k_{\text{cat}}/K_m \)-ratio was also found for peptides of varying length consisting of the N-terminal glutamine residue and alanine residues as substrates of human QC (Table 2). While human QC was more selective for substrates of a length up to a pentapeptide, there was no such a trend with the papaya QC. Human QC was less active toward a serine-containing peptide, indicating that also the nature of the substrate side chains of the amino acids close to the reaction center is of importance.

### Influence of Ionic Strength on Catalysis

To analyze the influence of ionic strength on the substrate specificity, the kinetic parameters for cyclization of several substrates were determined in presence and absence of 0.5 M KCl (Table 3). Interestingly, the specificity of both QCs
tested here can be regarded as putative substrates. In earlier studies, conversion of [Gln\(^1\)]-thyrotropin releasing-hormone ([Gln\(^1\)]-TRH) and [Gln\(^1\)]-gonadotropin releasing-hormone ([Gln\(^1\)]-GnRH) was shown for QC from bovine and porcine pituitary glands (Busby et al., 1987; Fischer and Spiess, 1987). In addition to the previously studied hypophysiotropic hormones, the following three potential physiological substrates of human QC were synthesized and tested: [Gln\(^1\)]-gastrin, [Gln\(^1\)]-neurotensin, and [Gln\(^1\)]-fertilization promoting peptide ([Gln\(^1\)]-FPP) (Table 3). The glutaminyl peptides were converted to the respective pyroglutamyl peptides with increasing specificity in order of their size, i.e., the largest peptide [Gln\(^1\)]-gastrin with 17 amino acids followed by [Gln\(^1\)]-neurotensin, [Gln\(^1\)]-GnRH, [Gln\(^1\)]-TRH and [Gln\(^1\)]-FPP.

Surprisingly, also the plant QC converted longer substrates with higher efficacy. Possibly there are secondary binding interactions between the substrate and the enzyme distant from the active site that may influence catalysis.

**Peptides Comprising Modified Amino Acids**

The specificity of the QCs was further analyzed with peptides that contain either a modified N-terminal glutaminyl residue or a modified penultimate amino acid. The conversion of these peptides was investigated qualitatively by MALDI-TOF mass spectrometry. Due to the cyclization of the glutaminyl residue and its analog, respectively, a mass difference of the substrate and the product of catalysis was detected. If ammonia was liberated equivalor, the conversion was also analyzed quantitatively using the spectrophotometric assay.

(1) **H-Gln-Lys(Gln)-Arg-Leu-Ala-NH\(^2\)** This branched peptide contains two glutaminyl residues bound to a lysyl residue via a peptide- and partial isopeptide bond. Human QC (Figure 5) and papaya QC (not shown) converted this compound apparently in an identical manner. Both glutaminyl residues were cyclized into pyroglutamic acid, and the consistent substrate conversion (Figure 5) indicate the lack of any preference for one residue. Thus, the specificity of the QCs for the differently bound glutaminyl residues seems not to differ fundamentally.

(2) **H-Gln(NMe)-Phe-Lys-Ala-Glu-NH\(^2\)** The methylated glutaminyl residue was only converted into a pyroglutamyl residue by papaya QC (not shown). An inhibition of human QC by the peptide was not detected, proving that the methylated residue is not recognized by human QC.

(3) **H-Glu(OMe)-βNA and H-Glu-βNA** Neither of these compounds were converted by papaya or human QC under the applied conditions. The O-methylated glutamic acid residue, however, showed a remarkable instability in both Tris and Tricine buffers leading to non-enzymatic cyclization, probably due to an increased polarity of the γ-ester. Furthermore, catalysis of both QC forms was not inhibited by the longer peptides H-Glu(OMe)-Phe-Lys-

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**Fig. 5** Formation of pGlu-Lys(pGlu)-Arg-Leu-Ala-NH\(^2\) from H-Gln-Lys(Gln)-Arg-Leu-Ala-NH\(^2\) through Catalysis by Human QC. Substrate conversion was followed by monitoring by a time-dependent change in the m/z ratio due to the liberation of ammonia. The sample composition was 0.5 mM substrate, 38 nM QC in 40 mM Tris/HCl, pH 7.7. At the times indicated, samples were moved, mixed with matrix solution (1:1 v/v) and the mass spectra recorded. A very similar dependence was observed in case of papaya QC. There was no substrate conversion in samples without QC (not shown).

(4) **H-Gln-cyclo(NE-Arg-Pro-Ala-Gly-Phe)** The conversion of H-Gln-cyclo(NE-Arg-Pro-Ala-Gly-Phe), which contains an intramolecular partial isopeptide bond was analyzed quantitatively, revealing \(K_m\) values of 240 ± 14 µM and 133 ± 5 µM for human and papaya QC, respectively. Due to the higher turnover number of conversion by the papaya QC (49.4 ± 0.6 s\(^{-1}\)) compared to the human QC (22.8 ± 0.6 s\(^{-1}\)), the plant enzyme exhibits with 372 ± 9 mM\(^{-1}\)s\(^{-1}\) an approximately 4-fold higher \(k_{cat}/K_m\) value than the human QC. Thus, the specificity constant is only slightly lower compared to substrates of similar size for papaya QC. The \(k_{cat}/K_m\) value for human QC was 95 ± 3 mM\(^{-1}\)s\(^{-1}\), being about one order of magnitude lower than with substrates of similar size (Table 1). Possibly, the N-terminal glutaminyl residue is less accessible by the enzyme active site due to steric hindrance of the bulky ring and this may have a stronger effect on catalysis by human QC than in case of the plant enzyme.

(5) **H-β homoGln-Phe-Lys-Arg-Leu-Ala-NH\(^2\)** The N-terminal β-homoglutaminyl residue was converted into a five-membered lactam ring by catalysis of the human and the papaya QC. The concomitant liberation of ammonia was analyzed spectrophotometrically and by MALDI-TOF mass spectrometry as described before. There was no liberation of ammonia detected when QC was omitted or boiled, proving enzymatic catalysis of the cyclization. Interestingly, the QCs from *C. papaya* (\(K_m = 3.1 ± 0.3\) mM, \(K_m = 2.3 ± 0.2\) mM, respectively) converted these substrates with higher efficacy than human QC.
ammonia is formed by conversion of nearly all QC substrates (Bateman, 1989). Originally, the assay was developed as a time-consuming discontinuous method. An increase in the amount of the auxiliary enzyme, however, allowed a continuous data monitoring, suitable to determine the kinetic parameters for various substrates. With the continuous assay, the kinetic parameters for conversion of about 40 glutaminyl peptides by plant and human QC were determined. Both glutaminyl cyclases share obvious similarities in their catalytic properties (Table 1); they exhibited the highest turnover numbers with substrates containing an aromatic amino acid residue in the penultimate position, and similar specificities were observed for peptides that consist of more than two amino acids. Furthermore, both QC forms revealed substrate inhibition only for the fluorogenic substrates, and showed an overall similar dependence on the ionic strength. Finally, the pH dependence of the specificity constant reveals a dependence of the overall catalysis on an unprotonated substrate amino group, and both QC forms catalyze the cyclization of L-[homoglutaminyl] residues with identical competence. Obviously, the QCs from plants and animals catalyze the cyclization of the glutaminyl residue with very similar efficiency and probably by an identical overall mechanism, i.e. non-covalent catalysis, by facilitating the intramolecular cyclization of the glutaminyl residue. This was already suggested for papaya QC (Gololobov et al., 1994). Initial data for the animal QC pointed to a nucleophilic influence by free thiol group(s), and subsequent formation of an acyl-enzyme during catalysis was suggested (Busby et al., 1987). More recently, however, human QC was shown to carry no free thiol groups, and the inhibition by iodoacetamide previously observed could be explained by a side reaction (Bateman et al., 2001; Schilling et al., 2002a). Non-cova lent catalysis by the human QC is also corroborated by the fact that this protein is assumed to possess a fold very similar to bacterial Zn-dependent aminopeptidases and that QC might be evolved from an ancestral aminopeptidase (Bateman et al., 2001). Interestingly, also the related aminopeptidase from Aeromonas proteolytica (AAP), like human QC, showed a remarkably improved substrate specificity towards longer peptide substrates.

Discussion

Based on the elucidation of the primary structures of the enzyme from papaya latex, bovine and human pituitary, several different properties were detected for QCs of plant and animal origin (Pohl et al., 1991; Song et al., 1994; Dahl et al., 2000). The sequence comparison revealed that the proteins have not much in common except the catalysis of pyroglutamyl formation. These findings prompted us to compare the substrate specificities of a plant and human QC in detail, in order to identify differences of the catalysis. Papaya QC, representing the first plant enzyme characterized, and human QC, representative for the highly homologous mammalian QCs, were chosen for this comparison. Papaya QC was purified from crude papain and the human enzyme was recombinantly expressed in P. pastoris. The recombinant QC was shown to have very similar characteristics and an identical specific activity compared to the highly homologous QC purified from bovine pituitary, suggesting that the recombinant enzyme resembles the native QC very closely (Schilling et al., 2002a).

Due to difficulties in detecting pyroglutamyl formation directly, coupled enzymatic assays have been develop ed. Detection of ammonia liberated in a coupled assay with glutamate dehydrogenase is most suitable, since the catalysis of pyroglutamyl formation. These findings prompted us to compare the substrate specificities of a plant and human QC in detail, in order to identify differences of the catalysis. Papaya QC, representing the first plant enzyme characterized, and human QC, representative for the highly homologous mammalian QCs, were chosen for this comparison. Papaya QC was purified from crude papain and the human enzyme was recombinantly expressed in P. pastoris. The recombinant QC was shown to have very similar characteristics and an identical specific activity compared to the highly homologous QC purified from bovine pituitary, suggesting that the recombinant enzyme resembles the native QC very closely (Schilling et al., 2002a).

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Table 4  Kinetic Parameters for Cyclization of Several Putative Physiological Substrates of Human QC.

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<tr>
<th>Substrate</th>
<th>Human QC</th>
<th>Papaya QC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (µM)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>[Gln$^1$]-Gastrin</td>
<td>31±1</td>
<td>54.1±0.6</td>
</tr>
<tr>
<td>[Gln$^1$]-Neurotensin</td>
<td>37±1</td>
<td>48.8±0.4</td>
</tr>
<tr>
<td>[Gln$^1$]-FPP</td>
<td>87±2</td>
<td>69.6±0.3</td>
</tr>
<tr>
<td>[Gln$^1$]-TRH</td>
<td>90±4</td>
<td>82.8±1.2</td>
</tr>
<tr>
<td>[Gln$^1$]-GnRH</td>
<td>53±3</td>
<td>69.2±1.1</td>
</tr>
</tbody>
</table>

Reactions were carried out as described in Table 1 (n.d., not determined; TRH, thyrotropin releasing-hormone; FPP, fertilization promoting peptide; GnRH, gonadotropin releasing-hormone).
possibly due to interactions between the P1',- and P2',-amino acid residue in the substrate and the enzyme (Wilkes et al., 1973). Therefore, it is tempting to suggest that animal QCs evolved from a Zn-dependent aminopeptidase keeping the secondary substrate interaction sites, but underwent structural rearrangements of the active-site geometry accompanied by a switch in the catalytic mechanism.

In contrast to human QC, papaya QC did not show such a distinct secondary specificity (Table 2). This holds true for a relaxed selectivity pattern concerning substrate length and composition, as well as the different $k_{\text{cat}}/K_m$-ratio for the partially cyclized substrate H-Gln-cyclo(Nr-Lys-Arg-Pro-Ala-Gly-Phe). Moreover, substrates containing proline in the second position and the ester substrate H-Gln-OrBu are well accepted by plant QC but only weakly by human QC. A further striking difference is the inability of human QC to convert the methylated glutaminyl residue of H-Gln(NMe)-Phe-Lys-Ala-Glu-NH$_2$ into a pyroglutamyl residue. This property seems to be attributable to N-terminal substrate binding, since this peptide did not inhibit human QC. Furthermore, neither cyclization of, nor inhibition by, the peptide H-Glu(NH$_2$)-Ser-Pro-Thr-Ala-NH$_2$ was detected for papaya QC. Human QC, however, was inhibited competitively by the N-terminal hydrazide peptide. The presented data suggest that both QCs differ in substrate binding. Obviously, there is more flexibility for substrate side-chain recognition by both enzymes. Interestingly, this is in great contrast to the strict requirement of an unprotonated substrate amino group for catalysis of both enzymes (Figure 3).

The conversion of the potential physiological substrates of human QC [Gln$^\gamma$]-gastrin, [Gln$^\gamma$]-neurotensin and [Gln$^\gamma$]-FPP, shown here for the first time, reflects the relatively broad substrate specificity of human QC. Possibly, the QC is physiologically active in the pGlu formation of most if not all N-terminally Gln-containing hormones. This implies that QC may occur in more tissues.

Differences, however, are evident in:
(i) the conversion of short peptide substrates and dipeptide surrogates,
(ii) the dependence of size and amino acid composition of the substrate and
(iii) the recognition mode of the modified $\gamma$-amide group of the N-terminal glutamine of the substrate.

To our knowledge, this is the first detailed analysis of substrate specificity of an animal QC and the first direct comparison of a plant and an animal QC. The continuous assay introduced allows the testing of a number of glutaminyl peptides revealing similarities in the overall potency for catalysis of glutamine cyclization, but also differences in the specificity of the QCs. Obviously, the differences are mainly based on dissimilar secondary binding sites for the substrates and a different recognition pattern of the N-terminal glutamine residue.

### Materials and Methods

**Materials**

Papaya QC was purified from crude papain as described previously (Schilling et al., 2002b). Human QC was expressed in *P. pastoris* and purified from the fermentation broth as described (Schilling et al., 2002a). The QC substrates H-Gln-Gln-OH, H-Gln-Glu-OH, H-Gln-Gly-OH, H-Gln-JNA, H-Gln-AMC and H-Gln-Ala-OH were purchased from Bachem (Bubendorf, Switzerland) or Senn Chemicals (Dielandsdorf, Switzerland). Pyroglutamate aminopeptidase from Bacillus amyloliquefaciens was supplied by Qiagen (Hilden, Germany). Glutamate dehydrogenase from bovine liver was purchased from Fluka (Seelze, Germany). NADH/H$^+$ and $\alpha$-ketoglutaric acid were obtained from Sigma (St. Louis, USA). All other chemicals were of analytical or HPLC grade.

**Peptide Synthesis**

**Oligopeptides**

Peptides were synthesized semiautomatically at a 0.5 mmol scale using a peptide synthesizer (Labortece SP650, Bachem) as previously described (Schilling et al., 2002a). Longer peptides were synthesized at a 25 µmol scale using the automated Symphony peptide synthesizer (Rainin Instrument Co., Emeryville, USA) as described (Manhart et al., 2003). For all peptide couplings, modified Fmoc-protocols of solid-phase peptide synthesis were employed using 2-[(1H-Benzo[c]triazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU; Novabiochem, Schwalbach, Germany)/base (disopropyl ethylamine or N-methyl-morpholine; Merck, Darmstadt, Germany) in case of difficult couplings N-[[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-N-methyl-methanammonium hexafluorophosphate N-oxide (4,5) (HATU; Applied Biosystems, Foster City, USA)/disopropyl ethylamine as activating reagents were used. After cleavage from the resin by a trifluoroacetic acid (TFA; Merck) containing cocktail, the crude peptides were purified by preparative HPLC with acid free solvents in order to avoid further cyclization of the N-terminal glutamine. Preparative HPLC was performed with a linear gradient of acetonitrile in water (5–40% or 65% acetonitrile over 40 min) on a 250–21 Luna RP18 column (Phenomenex, Torrance, USA). To confirm peptide purity and identity analytical HPLC and ESI-MS was applied.

**Conclusion**

The substrate specificity of human and papaya glutaminyl cyclase, enzymes that catalyze the same reaction but are not related in sequence, was analyzed in a comparative manner. Both enzymes reveal some analogy in their catalytic behavior, i.e., they show:

(i) a dependence of catalysis on an unprotonated N-terminal substrate amino group,
(ii) a similar specificity towards oligopeptide substrates and
(iii) similar requirements for the distance between $\alpha$-amino- and $\gamma$-amide group of the N-terminal glutaminyl residue.
Boc-protected peptides were deprotected by HCl/dioxane solution. Boc-Gln-Val-OMe were saponified by 1 N NaOH in dioxane. The C-terminal methylesters Boc-Gln-Tyr-OMe and mixed anhydride procedure by using isobutyl chlorocarbonate. Boc-protected dipeptides were synthesized applying standard H-Gln-Lys(Gln)-Arg-Leu-Ala-NH₂ yielding 76% yield.

The resin was filtered and washed with DMF and TFA. Following evaporation, the crude peptide was precipitated with ether giving 76% yield.

H-Gln-Lys(Gln)-Arg-Leu-Ala-NH₂ was synthesized according to standard Fmoc-procedure on Rink amide MBHA resin (Schilling et al., 2002a) using Fmoc-Lys(Fmoc)-OH as penultimate amino acid coupling. After deprotection of the two amino protecting groups of lysine with 20% piperidine (Merck) in DMF, 4 eq. Fmoc-Gln(Tri)-OH were coupled. Standard cleavage procedure resulted in 95% yield.

H-Gln(NMe)-Phe-Lys-Ala-Glu-NH₂ was synthesized starting from Fmoc-Glu-OfBu loaded on Fmoc-MI-AM (Novabiochem) resin. After swelling with DCM, the resin (0.5 g) was washed with DMF and deprotected with 20% piperidine solution in DMF. The resin was given into 5 ml DMF and 5 eq. Fmoc-Glu-OfBu, 5 eq. HATU and 10 eq. diisopropyl ethylamine were added subsequently and shaken for 6 hours. After filtration and washing, the product was cleaved according to standard TFA cleavage conditions. The peptide H-Gln(NMe)-Phe-Lys-Ala-Glu-NH₂ was synthesized as described (Schilling et al., 2002a). Fmoc-Gln(NMe)-OH was cleaved with HATU/disopropyl ethylamine overnight. The standard cleavage procedure resulted in a yield of 78% of the crude peptide.

H-Glu(OMe)-β-naphthylamide, H-Gln-Val-OH, H-Gln-Tyr-OH Boc-protected dipeptides were synthesized applying standard mixed anhydride procedure by using isobutyl chlorocarbonate (Merck). The C-terminal methyl esters Boc-Gln-Tyr-OMe and Boc-Gln-Val-OMe were saponified by 1 N NaOH in dioxane. The Boc-protected peptides were deprotected by HCl/dioxane solution for 10 min. After evaporation the residue was crystallized with several solvents giving 60 – 70% of a solid compound.

H-Gln-cyclo(Ne-Lys-Arg-Pro-Ala-Gly-Phe) The linear precursor Boc-Gln(Tri)-Lys-Arg(Pmc)-Ala-Gly-Phe-OH was synthesized on acid sensitive 2-chlorotrityl resin. Coupling was carried out using a standard protocol of Fmoc-strategy using Fmoc-Lys(Mtt)-OH. After cleavage with 3% TFA solution in DCM (10 times 5 min), the solution was neutralized with 10% pyridine in methanol (MeOH; Merck), washed 3 times with DCM and MeOH, evaporated to 5% of the volume and the crude peptide was precipitated with ice-cold water. Following, the crude peptide was cyclized using DCC/N,N-hydroxybenzotriazole (HOBt; Aldrich) activation. The crude peptide was dissolved in dry dichloromethane (0.2 mmol/50 ml), 0.2 mmol N-methylmorpholine and 0.4 mmol 1-hydroxybenzotriazole were added. This solution was added dropwise to a solution of 0.4 mmol dicyclohexylcarbodiimide in 250 ml dichloromethane at 0°C. The reaction was completed by stirring overnight at room temperature. After filtration of N,N'-dicyclohexylurea, the solvent was removed by evaporation. The residue was dissolved in ethyl acetate and washed several times with 1 N HCl, saturated solution of NaHCO₃ and water. The solution was dried over anhydrous Na₂SO₄, filtered and evaporated to dryness in vacuum. The crude product was purified by RP-HPLC yielding 12% of the cyclic peptide.

### Fluorometric Assays of QC

Human QC activity was evaluated using H-Gln-[JNA at 30°C, essentially as described (Schilling et al., 2002b). Briefly, the samples consisted of 0.2 mm fluorescent substrate, 0.1 U pyroglutamyl aminopeptidase in 0.05 M Tris/HCl, pH 8.0 containing 5 mM EDTA and an appropriately diluted aliquot of QC in a final volume of 250 µl. In case of papaya QC, the Tris buffer was substituted by 0.05 M Tricine/NaOH, pH 8.0. Excitation/emission wavelength was 320/405 nm. The assay reactions were initiated by addition of QC. Enzymatic activity was determined from the amount of released JNA calculated using a standard curve of JNA under assay conditions. One Unit is defined as the amount of QC catalysing the formation of 1 µmol pGlu-[JNA from H-Gln-[JNA per minute under the described conditions. Reaction conditions were the same in case of H-Gln-AMC, except that the excitation/emission wavelength was adjusted to 380/460 nm. The measurements were performed with a Novostar (BMG Labtechnologies, Offenburg, Germany) or a SpectraFluor Plus reader for microplates (Tecan, Männedorf, Switzerland).

### Spectrophotometric Assay of QC

QC activity was analyzed spectrophotometrically using a continuous assay that was established by adapting a discontinuous method (Bateman, 1989) using glutamate dehydrogenase as auxiliary enzyme. Samples consisted of the respective QC substrate, 0.3 mM NADH, 14 µM α-ketoglutaric acid and 30 U/ml glutamate dehydrogenase in a final volume of 250 µl. Reactions were started by addition of QC and pursued by monitoring of the decrease in absorbance at 340 nm for 8 – 15 min. The initial velocities were evaluated and the enzymatic activity was determined from a standard curve of ammonia obtained previously under assay conditions. All samples were measured at 30°C, using either the SpectraFluor Plus or the Sunrise reader for microplates (both from Tecan). Kinetic data were evaluated using GraFit software (version 5.0.4, for windows, Erithacus Software Ltd., Horley, UK).

### pH Dependence

The specificity rate constants (kcat/Km) at varying pH values were determined under first-order conditions, i.e., at substrate concentrations lower than Kᵥ, using H-Gln-[JNA as substrate. The reactions were measured either in a three-component buffer system that provides a constant ionic strength over a wide pH-range consisting of 0.025 M MES, 0.025 M acetic acid and 0.05 M NaCl. The buffers were titrated to the desired pH using HCl or NaOH. All measurements were performed with the Novostar reader (BMG Labtechnologies) for microplates at 23°C.

The pH-dependent rate data were evaluated by nonlinear regression utilizing GraFit software. Measured rates were fitted to the following equation:

\[
\frac{k_{cat}}{K_m} = \frac{k_{cat}}{K_m[\text{limit}]} \left[1 + 10^{(pH - \text{pK}_a)} \cdot 10^{(pH - \text{pK}_b)}\right],
\]
in which $K_1$ and $K_2$ are the dissociation constants of the catalytically important functional groups and $K_{cat}/K_{m(\text{limit})}$ is the pH-independent maximum rate constant.

**MALDI-TOF Mass Spectrometry**

Matrix-assisted laser desorption/ionization mass spectrometry was carried out using the Hewlett-Packard G2025 LD-TOF System (Palo Alto, USA) with a linear time-of-flight analyzer. The instrument was equipped with a 337 nm nitrogen laser, a potential acceleration source (5 kV) and a 1.0 m flight tube. Detector operation was in the positive-ion mode and signals were recorded and filtered using LeCroy 9350M digital storage oscilloscope linked to a personal computer. Samples (5 µl) were mixed with equal volumes of the matrix solution. For matrix solution we used DHAP/DAHC, prepared by solving 30 mg 2,6-dihydroxyacetophenone (Aldrich) and 44 mg diammominium hydrogen citrate (Fluka) in 1 ml acetonitrile/0.1% TFA in water (1/1, v/v). A small volume (~1 µl) of the matrix-analyte-mixture was transferred to a probe tip and immediately evaporated in a vacuum (Palo Alto, USA) with a linear time-of-flight analyzer. The instrumentation was carried out using the Hewlett-Packard G2025 LD-TOF System.

**Acknowledgments**

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**References**


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Identification of Human Glutaminyl Cyclase as a Metalloenzyme

POTENT INHIBITION BY IMIDAZOLE DERIVATIVES AND HETEROCYCLIC CHELATORS*

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Human glutaminyl cyclase (QC) was identified as a metalloenzyme as suggested by the time-dependent inhibition by the heterocyclic chelators 1,10-phenanthroline and dipyridochloroquinoline. The effect of EDTA on QC catalysis was negligible. Inactivated enzyme could be fully restored by the addition of Zn²⁺ in the presence of equimolar concentrations of EDTA. Little reactivation was observed with Co²⁺ and Mn²⁺. Other metal ions such as K⁺, Ca²⁺, and Ni²⁺ were inactive under the same conditions. Additionally, imidazole and imidazole derivatives were identified as competitive inhibitors of QC. An initial structure activity-based inhibitor screening of imidazole-derived compounds revealed potent inhibition of QC by imidazole N-1 derivatives. Subsequent data base screening led to the identification of two highly potent inhibitors, 3-[5-(1H-imidazol-1-yl)pentyl]-2-thioximidazolidin-4-one and 1,4-bis(2imidazol-1-yl)-methyl-2,5-dimethylbenzene, which exhibited respective Kᵢ values of 818 ± 1 and 295 ± 5 nM. The binding properties of the imidazole derivatives were further analyzed by the pH dependence of QC inhibition. The kinetics obtained pKᵢ values of 6.94 ± 0.02, 6.93 ± 0.03, and 5.60 ± 0.05 for imidazole, methylimidazole, and benzimidazole, respectively, match the values obtained by titrimetric pKᵢ determination, indicating the requirement for an unprotonated nitrogen for binding to QC. Similarly, the pH dependence of the kinetic parameter Kᵢ for the QC-catalyzed conversion of H-Gln-7-amino-4-methylcoumarin also implies that only N-termi-nally unprotonated substrate molecules are bound to the active site of the enzyme, whereas turnover is not affected. The results reveal human QC as a metal-de-pendent transferase, suggesting that the active site-bound metal is a potential site for interaction with novel, highly potent competitive inhibitors.

Glutaminyl cyclases (QC) (EC 2.3.2.5) are acyltransferases present in animals and plants that catalyze the conversion of N-terminal glutaminyl residues into pyroglutamic acid with the concomitant liberation of ammonia (Scheme 1). Several peptide hormones and proteins carry N-terminal pyroglutamyl residues. Previously, the formation of N-terminal pyroglutamyl peptides containing an N-terminal proline residue (13), whereas human QC was shown to be inactivated by 1,10-phenanthroline and reduced 6-methylpterin (8). Thus, we investigated the inhibiting potency of heterocyclic compounds from different structural classes. Among them, imidazole and structurally related compounds were found to be the most efficient competitive inhibitors of human QC. The data provide the first insights into enzyme/inhibitor interactions, offer clues for further optimization of the inhibitory structure, and reveal novel aspects of human QC catalysis.

EXPERIMENTAL PROCEDURES

Materials—Human QC was recombinantly expressed in Pichia pastoris and purified as described previously (10). Chemicals were purchased as follows. Glutamate dehydrogenase was from Fluka, pyroglutaminyl aminopeptidase came from Qiagen, H-Gln-AMC and H-Gln-Oh were from Bachem, NADH/H⁺ and α-ketoglutaric acid were from Sigma, and the imidazole derivatives were from Acros, Sigma.

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‡ The abbreviations used are: QC, glutaminyl cyclase; AMC, 7-amino-4-methylcoumarin; Mes, 4-morpholinethanesulfonic acid.

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Inhibitory Assay—For inhibitor testing, the sample composition was the same as described above, except for the addition of the inhibitory compound. For rapid inhibitor screening, samples contained up to 4 mM of the respective imidazole derivative and a substrate concentration equal to the $K_m$ value of the test substrate. For detailed investigations of the inhibition and determination of $K_i$ values, the influence of the inhibitor on the auxiliary enzymes was investigated first. In no case was an influence on one of the auxiliary enzymes detected, enabling the reliable determination of the QC inhibition. The inhibition constants were evaluated by fitting the data of the obtained progress curves according to the general equation for competitive inhibition using

$$
\frac{1}{V} = \frac{1}{V_0} + \frac{K_i [I]}{V_0 K_i + [I]}
$$

where $V$ is the observed velocity, $V_0$ the maximum velocity in the absence of inhibitor, $[I]$ the inhibitor concentration, and $K_i$ the inhibition constant.

$pH$ Dependence—For the investigation of the pH dependence of QC catalysis and inhibition, the previously developed fluorometric assay was used (14). Determinations were carried out at 30 °C in a buffer originally used by Ellis and Morrison consisting of 0.08 M acetic acid, 0.06 M Mes, and 0.12 M Tris pH 8.0. The buffer provides a constant ionic strength over the entire pH range chosen. Additionally, the activity of human QC acting on H-Gln-AMC has been shown to be quite independent from variations in ionic strength. The resulting pH dependence data were fitted to single dissociation models for the inhibitors or to equations that account for two dissociating groups in the case of the kinetic parameters.

Inhibition constants of imidazole derivatives in the human QC catalyzed reaction

- **Core structures**
  - Imidazole: 103 ± 4
  - Benzimidazole: 138 ± 5

- **N-1 derivatives**
  - 1-Benzylimidazole: 7.1 ± 0.3
  - 1-Methylimidazole: 30 ± 1
  - 1-Vinylimidazole: 49 ± 2
  - Oxalic acid diimidazolidide: 78 ± 2
  - N-Acetylimidazole: 107 ± 3
  - N-(Trimethylsilyl)-imidazole: 167 ± 7
  - N-Benzylimidazole: 174 ± 7
  - 1-Oxo-2-phenethyl-imidazole: 184 ± 5
  - 1-(Aminopropyl)-imidazole: 410 ± 10
  - 1-Phenylimidazole: No inhibition
  - 1,1-Sulfonylimidazole: No inhibition

- **C-4 (5) derivatives**
  - N-(ω-acetylhistamine: 17 ± 1
  - 1-Histidinamide: 560 ± 40
  - H-His-Trp-OH: 600 ± 30
  - 1-Histidinol: 1550 ± 120
  - 1-Histidine: 4400 ± 200
  - 4-Imidazole-carboxaldehyde: 7600 ± 700
  - Imidazol-4-carboxylic acid methylester: 14500 ± 600

- **C-4,5 derivatives**
  - 5-Hydroxymethyl-4-methylimidazole: 129 ± 5
  - 5-Amino-3H-imidazole-4-carboxylic acid amide: 15500 ± 500
  - 4,5-Diphenylimidazole: No inhibition
  - 4,5-Dicyanimidazole: No inhibition

- **C-2 derivatives**
  - 2-Methylbenzylimidazole: 165 ± 4
  - 2-Ethyl-4-methylimidazole: 580 ± 40
  - 2-Aminobenzimidazole: 1800 ± 100
  - 2-Chloro-1H-benzimidazole: No inhibition

**TABLE I**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ value</th>
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<tr>
<td>Imidazole</td>
<td>103 ± 4</td>
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<tr>
<td>Benzimidazole</td>
<td>138 ± 5</td>
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<tr>
<td>1-Benzylimidazole</td>
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<tr>
<td>1-Methylimidazole</td>
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<tr>
<td>1-Vinylimidazole</td>
<td>49 ± 2</td>
</tr>
<tr>
<td>Oxalic acid diimidazolidide</td>
<td>78 ± 2</td>
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<tr>
<td>N-Acetylimidazole</td>
<td>107 ± 3</td>
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<tr>
<td>N-(Trimethylsilyl)-imidazole</td>
<td>167 ± 7</td>
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<tr>
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<td>1-Oxo-2-phenethyl-imidazole</td>
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<td>1-(Aminopropyl)-imidazole</td>
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<td>1-Phenylimidazole</td>
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<tr>
<td>1,1-Sulfonylimidazole</td>
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<tr>
<td>N-(ω-acetylhistamine)</td>
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<td>1-Histidinamide</td>
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<td>2-Aminobenzimidazole</td>
<td>1800 ± 100</td>
</tr>
<tr>
<td>2-Chloro-1H-benzimidazole</td>
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</tr>
</tbody>
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**FIG. 1**

Lineweaver-Burk plots for human QC catalyzed cyclization of H-Gln-AMC in presence of various concentrations of imidazole between 0.03 and 1 mM. The inset shows a secondary plot of the obtained slopes of the Lineweaver-Burk evaluation versus the inhibitor concentrations. The conditions were 0.05 M Tris/HCl, pH 8.0, containing 5 mM EDTA at 30 °C.

**SCHEME 2**

The constitution of the imidazole ring (A) and an imidazole N-1 derivative (B).
Reactivation experiments were performed at room temperature for 15 min using Zn$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, Ca$^{2+}$, K$^{+}$, and Co$^{2+}$ ions at concentrations of 1, 0.5, and 0.25 mM in 0.025 M Bis-Tris, pH 6.8, containing 0.5 mM EDTA. QC activity assays were performed in 0.05 M Tris/HCl, pH 8, containing 2 mM EDTA to avoid a rapid reactivation by the traces of metal ions present in buffer solutions.

**RESULTS**

Inhibition by Imidazole—Because neither glutamic acid dehydrogenase nor pyroglutamyl aminopeptidase were inhibited by imidazole in the concentration range used, both the fluorometric as well as the spectrophotometric assay could be applied. The Lineweaver-Burk plot of the fluorometric assay data (Fig. 1) reveals competitive inhibition by imidazole (inset in Fig. 1). Thus, imidazole binds in the active site completely blocking substrate conversion. The $K_i$ values of $10^3$ and $10^4$ obtained with the fluorometric and chromogenic assay, respectively, match very well. Interestingly, benzimidazole inhibits human QC similarly as does imidazole, also exhibiting linear competition and a $K_i$ value of $10^3$. $N$-$ω$-acetylated histamine proved to be a potent inhibitory compound. Small substituents in both positions (4 and 5) seemed to have only minor effects on binding, as indicated by the similar inhibition constants of 5-hydroxymethyl-4-methylimidazole and imidazole itself. Larger and more bulky groups attached to these sites diminished or abolished binding of the compounds to the enzyme. However, some of the substituents of the tested imidazole derivatives are known to exert negative inductive or mesomeric effects, thereby reducing the electron density within the imidazole ring. This could also contribute to poorer binding. The different $K_i$ values detected for L-histidine and histidinamide also indicate an influence of the charge of the inhibitors on binding. Evidence for electrostatic repulsion of charged substrates was observed previously during an investigation of the substrate specificity of QC, i.e. glutaminamide was readily converted to products by human QC, but glutamine was not cyclized (24).

C-4 (5) and C-4,5 Derivatives—The compounds with substitutions either in the constitutionally equivalent 4 or 5 position of the imidazole ring or in both positions showed reduced inhibitory activity toward human QC. In contrast, $N$-$ω$-acetylated histamine proved to be a potent inhibitory compound. Small substituents in both positions (4 and 5) seemed to have only minor effects on binding, as indicated by the similar inhibition constants of 5-hydroxymethyl-4-methylimidazole and imidazole itself. Larger and more bulky groups attached to these sites diminished or abolished binding of the compounds to the enzyme. However, some of the substituents of the tested imidazole derivatives are known to exert negative inductive or mesomeric effects, thereby reducing the electron density within the imidazole ring. This could also contribute to poorer binding. The different $K_i$ values detected for L-histidine and histidinamide also indicate an influence of the charge of the inhibitors on binding. Evidence for electrostatic repulsion of charged substrates was observed previously during an investigation of the substrate specificity of QC, i.e. glutaminamide was readily converted to products by human QC, but glutamine was not cyclized (24).

C-2 derivatives—All derivatives tested showed a diminished binding to the active site of QC relative to imidazole. Obviously, there is a strong impact on proper binding by any additional atom in this position. For instance, the simple addition of a methyl group to form 2-methyl-benzylimidazole reduces the
inhibition constant of the interaction by about one order of magnitude. A very similar relation becomes evident comparing the $K_i$ values for benzimidazole and 2-amino-benzimidazole.

**N-1 Derivatives**—Among the imidazole derivatives tested as inhibitors of human QC, most compounds that had reduced $K_i$ values compared with imidazole contained modifications at the N-1 nitrogen atom (Table I). Interestingly, only minor changes in the N-substituent were necessary for substantial loss of inhibitory power. This can be seen when comparing 1-benzylimidazole, 1-benzoylimidazole, and phenylimidazole as QC inhibitors. The data suggest, however, that steric hindrance for QC binding of N-1 derivatives is marginal, opening up the possibility for the development of even more potent QC inhibitors by structure optimization of N-1 modified imidazole compounds.

**Compound Data Base Screening**—The apparent improvement of the inhibitory power obtained by N-1 substitutions of the imidazole ring allowed us to identify highly potent inhibitors of QC by data base screening. Some of the most potent inhibitors are shown in Table II. In fact, the observed inhibition constants are one order of magnitude lower as compared with those determined in the initial structure-activity relationship experiments (Table I). This approach led finally to the identification of hit compounds exhibiting $K_i$ values of the QC inhibition in the nM range.

**Effect of 1,4 and 1,5 Derivatization**—The inhibition constants obtained for the 4(5)-substituted imidazole derivatives already indicated that there are restrictions for efficient binding to the enzyme. An individual contribution of position 4 and 5, however, were undetectable, because both are identical with respect to substitutions at one carbon. The individual effect of substitutions in position 4 and 5 was analyzed by comparing the inhibitory constants of L-histamine and the two intermediates in the biological degradation of histamine, 1-methyl-4-histamine, and 1-methyl-5-histamine (Table III). Interestingly, whereas methylation of one nitrogen of histamine forming 1-methyl-5-histamine improved the inhibitory activity considerably, methylation of the other nitrogen (1-methyl-4-histamine) led to a near total loss of inhibitory potential. Thus, steric hindrance by the carbon atom adjacent to the basic nitrogen seems to occur, which provides further support for the key role of the basic nitrogen in binding of the imidazole derivatives to the enzyme.

**pH Dependence**—The role of the basic nitrogen of imidazole was further characterized through an investigation of the pH-dependence of QC inhibition. Because of a limited catalytic activity as well as the reduced stability of the auxiliary enzyme pyroglutamyl aminopeptidase, this analysis was limited to a pH range between pH 5.5 and 8.5. Because imidazole has a $pK_a$ value near neutrality, however, this range was assumed to be sufficiently wide for inspecting the influence of protonation and deprotonation of the inhibitor. The inhibition of the QC-catalyzed reaction showed a strict dependence on the pH value (Fig. 2). With decreasing pH, the $K_i$-value of imidazole increased drastically, exhibiting a 25-fold increase when moving from pH 8 to 5.5. Furthermore, in the basic pH region, $K_i$ was constant, suggesting that the potency of QC inhibition depends on deprotonation of the imidazole derivatives. This was also corroborated by fitting of the data to a single dissociation model (Fig. 2). The dissociating group influencing the inhibitory properties of imidazole was characterized by a $pK_a$ value that is in excellent agreement with the $pK_a$ of the basic nitrogen of imidazole (Table IV). Similar pH dependences were obtained for QC in-

---

**TABLE III**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$-value (µM)</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-histamine</td>
<td>850 ± 40</td>
<td><img src="image1" alt="Structure of L-histamine" /></td>
</tr>
<tr>
<td>1-methyl-5-(β-aminoethyl)-imidazole</td>
<td>120 ± 4</td>
<td><img src="image2" alt="Structure of 1-methyl-5-(β-aminoethyl)-imidazole" /></td>
</tr>
<tr>
<td>1-methyl-4-(β-aminoethyl)-imidazole</td>
<td>n.i.</td>
<td><img src="image3" alt="Structure of 1-methyl-4-(β-aminoethyl)-imidazole" /></td>
</tr>
</tbody>
</table>

Assays were carried out as described in Table I. Another designation of the derivatives is tele-methylhistamine. They are in vivo occurring metabolites of histamine.
hibition by benzimidazole and 1-methylimidazole (Fig. 2). For both compounds, the kinetically determined $pK_a$ values compare well with the $pK_a$ values determined by titration (Table IV). The dependence of the kinetic parameters $K_m$ and $k_{cat}$ on the pH-value was also analyzed (data not shown). Whereas the turnover number for conversion of H-Gln-AMC was not affected in the pH range between 5.5 and 8.5, the Michaelis constant showed a simple pH dependence with an optimum between pH 7.5–8, tending to increase in the acidic and basic pH region. The pH dependence of $K_m$ revealed a slope of 1 in the acidic pH range, reflecting the presence of a single underlying dissociative group. The kinetically determined $pK_a$ value in the acidic range was nearly identical to the $pK_a$ of the substrate.

**Table IV**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Parameter</th>
<th>$pK_a$ kinetic determination</th>
<th>$pK_a$ titrimetric determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidazole</td>
<td>$K_m$</td>
<td>6.94 ± 0.02</td>
<td>6.94 ± 0.003</td>
</tr>
<tr>
<td>Benzimidazole</td>
<td>$K_m$</td>
<td>5.60 ± 0.05</td>
<td>5.50 ± 0.010</td>
</tr>
<tr>
<td>1-Methylimidazole</td>
<td>$K_m$</td>
<td>6.93 ± 0.03</td>
<td>7.00 ± 0.003</td>
</tr>
<tr>
<td>H-Gln-AMC</td>
<td>$K_{M(E)}$</td>
<td>6.81 ± 0.04</td>
<td>6.830 ± 0.010</td>
</tr>
<tr>
<td></td>
<td>$K_{M(H)}$</td>
<td>8.60 ± 0.10</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 3.** Inhibition of human QC by metal-chelating reagents. Concentration dependence of inhibition by 1,10-phenanthroline (circles) and EDTA (triangles) is shown. Residual activity of QC in the presence of either compound was determined directly after the addition (dotted traces) or preincubation of QC with the respective reagent for 15 min at 30 °C (continuous line).

**Fig. 4.** Reactivation of human QC with monovalent and divalent metal ions. QC was nearly inactivated by the addition of 2 mM dipicolinic acid in 50 mM Bis-Tris, pH 6.8. Subsequently, the enzyme was subjected to dialysis against 50 mM Bis-Tris, pH 6.8, containing 1 mM EDTA. Reactivation of the enzyme was achieved by incubation of the inactivated enzyme sample with metal ions at a concentration of 0.5 mM in the presence of 0.5 mM EDTA to avoid an unspecified reactivation by traces of metal ions present in buffer solutions. Controls are given by enzyme samples that were not inactivated but also dialyzed against the EDTA solution as the inactivated enzyme (+EDTA) and by enzyme samples that were dialyzed against buffer solutions without added EDTA (−EDTA).

**Discussion**

After a more detailed comparison, human QC does not seem to have much in common with its counterpart from the plant *Carica papaya* except for the catalyzed reaction. In a recent study of substrate specificity, we found a relatively similar proficiency for glutaminyl cyclization by both enzymes (24). However, differences were observed in binding and conversion of peptides bearing the modified N-terminal glutaminyl residues γ-hydrazide or γ-methylamide. Although human QC is inhibited by the hydrazide derivative (not papaya QC), only the methylamide derivative is recognized and cyclized by the plant enzyme. These results have already suggested differences in the recognition modes of the substrate glutaminyl residue by both enzymes. Additionally, we were unable to detect any inhibition of human QC by peptides containing N-terminal proline, which strongly inhibit papaya QC (13). Furthermore, in striking contrast to the prominent inhibition of human QC by imidazole derivatives, papaya QC was not influenced at all by any of these compounds.

Similarly as with metal-dependent aminopeptidases, human QC is inhibited by imidazole, 1,10-phenanthroline, and dipicolinic acid (17–19). In contrast to EDTA, these compounds all show a planar structure, possibly enabling the interaction with
the active site-bound metal ion. Because of the complete reactivation by the addition of Zn$^{2+}$ ions to apo QC, one can conclude that human and probably all mammalian QCs are Zn$^{2+}$-dependent. Recently, a relationship of the tertiary structure of human QC and the aminopeptidase from Vibrio proteolyticus, a prominent member of the clan MH family M28 of metallopeptidases, was proposed (9). Comparing the sequence of human QC with those of two members of the clan MH (Fig. 5), the binding motif His-Asp-Glu-Asp-His of the two Zn$^{2+}$ ions present in this clan of hydrolases is also conserved in human QC. Furthermore, as shown in another study (9), modification of two of the identified histidine residues, His-140 and His-330, which are probably necessary for metal binding (Fig. 5), leads to a complete loss of catalytic activity. These data further substantiate the fact that mammalian QCs evolved from an ancestral metal hydrolase and that at least one of the metal binding sites is conserved. It remains unclear, however, how the zinc ion(s) is involved in the catalysis of human QC. In the metallopeptidases, the catalytic mechanism leads to the formation of a non-covalent tetrahedral intermediate after the attack of a zinc-bound water molecule on the carbonyl group of the scissile bond. Either in parallel or subsequently, zinc binding stabilizes the oxanion of the formed tetrahedral intermediate. Zinc ions increase the nucleophilicity of the peptide bond-

**Fig. 5. Sequence alignment of human QC and other M28 family members of the metallopeptidase clan MH.** Multiple sequence alignment was performed using ClustalW at ch.EMBnet.org with default settings. The conservation of the zinc-ion ligating residues is shown for human QC (hQC; GenBank™ number X71125), the zinc-dependent aminopeptidase from Streptomyces griseus (SGAP; Swiss-Prot number P80561), and within the N-acetylated α-linked acidic dipeptidase (NAALADase I) domain (residues 274–587) of the human glutamate carboxypeptidase II (hGCP II; Swiss-Prot number Q04609). The amino acids involved in metal binding are set in **boldfaced type** and *underlined*. In the case of human QC, these residues are the putative counterparts to the peptidases. The shaded histidines (His-140 and His-330) indicate residues that were identified as being essential for QC catalysis (9).
attacking water molecule and polarize the scissile bond, making it susceptible to nucleophilic attack during transition state formation, with its progression to and the subsequent collapse of the tetrahedral intermediate followed by amid bond cleavage (20).

For the catalysis by human QC, the pH-dependence of substrate binding suggests that perhaps the metal ion could interact with the nitrogen of the N-terminal amino function of the substrate. Because QC catalyzes an intramolecular cyclization, the proper positioning of the substrate nitrogen in close proximity to the \( \gamma \)-carbonyl carbon is probably of essential catalytic importance. On the other hand, it seems likely that a metal ion in the active site of QC acts by polarizing the \( \gamma \)-amide group of the substrate glutaminyl residue, simultaneously stabilizing the oxanion formed by the nucleophilic attack of the \( \alpha \)-amino group of the substrate. Because QC catalyzes an intramolecular cyclization, the occupation of such a binding site with the active-site zinc ion with the \( \gamma \)-hydradise residues. Furthermore, the interaction of one active site zinc ion with the \( \alpha \)-amino group of the substrate and the polarization of the carbonyl group of the scissile peptide bond by another are proposed steps in the catalysis of the related aminopeptidase from \( V. \ proteolyticus \) (20). Accordingly, the metal ion(s) of QC might serve as a binding site for the imidazole-derived inhibitors and the substrate, with an unprotonated nitrogen interacting in analogy to the related peptidase.

In contrast to the aminopeptidase from \( V. \ proteolyticus \), however, increasing \( Zn^{2+} \) concentrations in QC assays (0.1 mtl and higher) considerably reduce QC activity, which also was observed in previous studies (3). Thus, it needs to be clarified whether QC also possesses two metal ions bound to the apoenzyme or whether, during evolution from an aminopeptidase to a metalloaminopeptidase group, including remodeling of the active site upon occupation of such a site high concentrations of \( Zn^{2+} \) ions may block the intramolecular reaction of the substrate.

It should be also noted, in this respect, that we could not detect any proteolytic activity of QC. Moreover, 1-butaneboronic acid and peptide thiols (22, 29), potent inhibitors of \( V. \ proteolyticus \) aminopeptidase, did not inhibit human QC, supporting potential changes in the active site geometry of QC compared with the aminopeptidases. Because there have been extensive rearrangements during the evolution of the zinc hydrolase group, including remodeling of the active site upon changes in zinc ligation (20, 21), only the solution of the protein structure will finally clarify the binding modes of substrate and inhibitor.

In contrast to its mammalian counterparts, papaya QC is not inhibited by metal chelators, suggesting a metal-independent mechanism. However, for the cyclization reaction, the nitrogen of the \( \alpha \)-amino group of the glutaminyl residue also needs to be deprotonated (Scheme 1), and both enzymes show a similar catalytic proficiency of catalysis. How the same catalytic reaction of such structurally divergent protein catalysts is maintained will remain obscure until the solution of the three-dimensional structures of both proteins.

In summary, we present here the first systematic structure-activity study of inhibitors for a mammalian QC. Because there is no reliable active site model for any QC available to date, there was only minimal information to identify the structural features that need to be incorporated into potent QC inhibitors. Besides the identification of \( N1 \)-imidazole derivatives as highly potent competitive inhibitors, the results revealed human QC as a metal-dependent enzyme as shown by the following: (a) the pH-dependence of inhibition by imidazole and imidazole derivatives; (b) the inactivation of QC by the metal-chelating reagents 1,10-phenanthroline and dipicolinic acid; (c) the reactivation of the QC-apoenzyme by bivalent metal ions; and (d) the conservation of metal-binding residues in the primary structure of QC. Finally, the observed impact of structural modifications of the imidazole derivatives on their QC-inhibitory potency can serve as a starting point for further, rationally driven inhibitor designs.

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REFERENCES

Continuous Assays of Glutaminyl Cyclase: From Development to Application

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Abstract

Glutaminyl cyclase (QC, EC 2.3.2.5) catalyses the formation of pyroglutamyl residues from glutamine at the N-terminus of peptides and proteins. In previously applied assays, QC activity was determined by either analysing the products formed using HPLC coupled with photometric or fluorometric detection, radioimmunoassay, or by detecting the release of ammonia spectrophotometrically. Although these methods are sensitive, they are all discontinuous and therefore time-consuming and laborious. To conduct detailed kinetic investigation of QC-catalysis, we developed coupled continuous assays suitable for microplates which allow now convenient determination of QC activity. The methods either use pyroglutamyl aminopeptidase or glutamate dehydrogenase as auxiliary enzymes, which results in the liberation of chromophores or fluorophores such as pNA, AMC, βNA or in the conversion of the chromophore NADH/H+ into NAD+, respectively.

The assays were applied in various enzyme isolation and characterization studies, using crude protein solutions as well as purified enzyme in pH-dependence, substrate and inhibitor specificity investigations. Depending on the respective analytical task, both assays complement each other. Therefore, different enzymatic properties could be explored in more detail. Since the employed strategy of assay development could be of interest also for the analysis of other enzymes, the methods are described here in a comprehensive manner.
Introduction

Several peptides and proteins contain pyroglutamic acid at their N-terminus. Initially, pyroglutamyl formation was assumed to result from a spontaneous cyclisation reaction of a \( N \)-terminal glutamine or glutamic acid residue. However, specific enzymatic conversion of glutamine by glutaminyl cyclase (EC 2.3.2.5) has been discovered in plant and animal tissues [1,2,3,4]. Although a QC was first explored in papaya latex, its physiological function in the plant is still enigmatic. More recently, however, QCs were also identified in several other plant species, suggesting a general physiological significance of this protein [5]. In contrast to plant QC, several physiological substrates and products of mammalian QC-activities could be identified. Pyroglutamic acid is present, for instance in the hormones Thyrotropin releasing hormone (TRH), gonadoliberin (GnRH) and gastrin, neurotensin and chemokines of the monocyte chemotactic protein (MCP) family. The formation of this \( N \)-terminal 5-oxoproline residue has shown to cause the bioactive structure of the hormones and to improve their stability towards N-terminal proteolysis [6,7,8]. Interestingly, plant and animal QCs seem to be very similar at a first glance. Both enzyme forms are expressed via the secretory pathway, carry carbohydrates and are monomeric proteins with similar molecular masses of 33-40 kDa [5,9,10]. Furthermore, all QCs are strictly specific for L-glutamine in the \( N \)-terminal position of the substrates and their kinetic behaviour was found to obey the Michaelis-Menten equation [10,11,12]. The primary and secondary structures of the QCs from \( C. \ papaya \) and that of the highly conserved QC from mammals, however, did not reveal homology [5,13,14]. Due to this apparent divergence, a detailed comparison of the catalytic properties of the QC forms could be helpful for deepening the understanding of pyroglutamyl formation and to identify, whether the different QC forms catalyse the pyroglutamyl formation by the same mechanism.

A detailed enzymatic characterisation of QC catalysis and inhibition, however, was hampered by the lack of handy assays. In previously applied methods, QC activity was determined by either
analysing the products formed using HPLC linked to photometric or fluorimetric detection [11,10] or radioimmunoassay [3,15] using antibodies directed against TRH, or by detecting the release of ammonia spectrophotometrically in a coupled enzymatic assay [16]. Although the methods are sensitive, they are all discontinuous and therefore time-consuming and laborious. Furthermore, some of these methods can be only applied for one certain substrate, thus hampering detailed substrate specificity studies. In another approach, the change in absorbance occurring due to the formation of an intramolecular amid bond during $N$-terminal pyroglutamyl formation by QC is detected [17]. Although this assay allows a continuous data monitoring, the observed changes in absorbance are very small, making the assay insensitive. Furthermore, due to measuring the absorbance change at 220 nm, at the wavelength characteristic for the $n \rightarrow \pi^*$ electron transition of peptide bonds, enzyme activity in crude samples cannot be determined because of the huge background. Accordingly, also the high initial absorbance of large peptides hinders the determination of catalytic parameters for such QC substrates.

Due to these disadvantages, the development of new assays was triggered that allow a) the convenient and fast determination of QC activity, making it suitable during protein purification and characterization and b) to easily determine the specificity of QCs for an assortment of substrates of different size and structure. This flexibility was obtained by developing coupled continuous assays that utilize different auxiliary enzymes. The inability of detecting the intramolecular amid transferase reaction in buffered systems could be compensated by well-observable coupled reactions, enabling detailed QC-characterisation studies such as substrate and inhibitor specificity, influence of ionic strength and pH-dependence of the kinetic parameters.
**Coupled enzymatic assays - theoretical considerations.** Enzyme catalysed reactions are most often analysed using spectrophotometric or fluorimetric detection, since the detectors, i.e. the photometers or fluorimeters, are relatively inexpensive and present in nearly all life science laboratories. However, many enzyme catalysed reactions cannot be monitored directly, since substrate conversion does not result in a change of the absorbance or fluorescence characteristics, as for instance the case in kinase-, phosphatase- or many transferase-catalysed reactions. Therefore, coupled enzymatic assays were established, using an auxiliary reaction that results in a change in absorbance or fluorescence. In the coupled reaction, one of the products of the reaction that should be analysed is consumed. The simplest case, which is also valid for the assays described below, can be represented schematically

\[
\begin{align*}
A & \xrightarrow{k_1} B \\
\text{primary enzyme} & \quad \text{auxiliary enzyme} \\
B & \xrightarrow{k_2} C
\end{align*}
\]

For a reliable assay, the following assumptions have to be fulfilled [18]: a) \(k_1\) represents a zero-order rate constant, i.e. the concentration of A does practically not change during the observed reaction time, b) the second reaction is irreversible, and c) \(k_2\) is a rate constant of first order, which requires that the concentration of B is always much lower than the Michaelis constant of the auxiliary enzyme for B ([B]<< \(K_B\)). Based on these assumptions, the rate equation focussed on formation and consumption of B, which is the prerequisite of the observed spectroscopic changes, is

\[
\frac{d[B]}{dt} = k_1 - k_2[B] 
\]
which provides after integration

\[ [B] = \frac{k_1}{k_2} (1 - e^{-k_2 t}) \]  \hspace{1cm} (2)

As can be seen from this equation, if time runs to infinity \((t \rightarrow \infty)\), a constant concentration of B is reached, the so-called steady state concentration \([B_{ss}]\), characterised by a linear progress of the formation of C. In a practical view, progress curves are usually indistinguishable from linearity, if 95 % of the intermediate steady-state concentration is reached, which is sufficient for providing reliable results. The time to reach this state is characterised by a “lag phase”, the progress curve shows an exponential increase. After rearrangement of equation 2, substitution of \(-V_{max2} t/K_B\) for \(-k_2 t\) and \([B_{ss}]\) for \((k_1/k_2)\), the following equation is obtained, which offers the opportunity to calculate the time until reaching assay conditions that provide progress curves indistinguishable from linearity

\[ V_{max2} = \frac{-K_B \ln(1 - [B]/[B_{ss}])}{t^*} \]. \hspace{1cm} (3)

In this equation, \(t^*\) denotes the time to reach a certain fraction of \([B_{ss}]\) (i.e., \([B]/[B_{ss}]\)), which is dependent on the concentration and specificity of the auxiliary enzyme for its substrate, indicated by \(V_{max2}/K_B\). Therefore, with knowledge of the specificity of the respective auxiliary enzyme, one can calculate the amount of protein required to obtain a reliable assay, without consuming excessive protein quantities. As follows, equation 3 was applied for the development of two different continuous assays for determination of QC activity.

**Coupling QC to Pyroglutamyl Aminopeptidase (pGAP) catalysis - development.** Coupling the cyclising activity of QC to a peptidase was accomplished by use of dipeptide surrogates that are prone to cleavage after conversion by QC. Accordingly, potential assay substrates possessing \(N-\)
terminal glutamine are Gln-pNA, Gln-βNA or Gln-AMC. After cyclisation by QC, the respective intermediates pGlu-pNA, pGlu-βNA or pGlu-AMC are hydrolysed by pGAP, liberating a chromophoric or fluorogenic group. Since the spectrophores are liberated in equimolar amounts to the glutaminyl-substrate converted, QC-activity can be calculated from standard curves. The reactions are exemplified for the turnover of Gln-βNA in Figure 1.

For assay development, the bacterial pyroglutamyl aminopeptidase from *Bacillus amyloliquefaciens* was chosen. This well-characterised cysteine protease shows a broad substrate specificity, suitable stability and is commercially available. With regard to specificity, the potential intermediates in QC assay have shown to be among the best substrates of this enzyme [19,20,21,22]. The time to reach virtual steady state conditions in the QC assay, if 1 U/ml auxiliary enzyme is applied, were calculated according to equation 3 using the available specificity data of pGAP (table 1). Due to the relatively low specificity of pGAP towards the intermediate pGlu-pNA, the time until observation of linear progress curves is approximately 2 min for the chromophoric substrate Gln-pNA. In contrast, when using Gln-βNA as QC substrate, virtual steady state conditions are observed within one second after initiation of the reaction caused by the high specificity of pGAP for the intermediate pGlu-βNA.

In fact, linear progress curves were observed according to such calculation. They are exemplified for Gln-βNA and Gln-AMC in Figure 2. For all substrates, there was a linear relationship between the QC concentration and the observed rate, indicating the linear dependence of the assay on conversion of the QC substrate (not shown). Finally, the assay could be applied for recombinant human or mouse and purified papaya QC. The now possible characterisation studies enabled the comparison of the QC forms concerning differences and similarities of their catalysis. Due to the shorter lag times observed with the fluorogenic substrates (Table 1), the assays using Gln-AMC or Gln-βNA provide a higher flexibility. Small alterations in the activity of the auxiliary enzyme do not affect the assay, because the auxiliary enzyme activity is still excessive to provide reliable results, i.e. the “lag times” are always shorter than the time required for starting the reaction and
mixing of the samples. Therefore, most of the characterising studies shown below were carried out using the fluorogenic substrates.

**Coupling QC to Pyroglutamyl Aminopeptidase (pGAP) catalysis – application.** Applications of spectroscopic enzyme assays range from the identification of enzyme activity in tissues, quantification of enzymatic activity during protein purification, protein characterisation in terms of pH-dependence of catalysis, substrate specificity and for inhibitor screening. Recently, we applied the assay during purification of papaya QC [23]. Although the continuous data monitoring already accelerated the enzyme determination during the purification procedure, its application is much more important during characterisation studies, since many assay reactions have to be performed, thus favouring the continuous assays. Moreover, only the analysis of kinetic parameters investigating a wide substrate concentration range makes it possible to detect differences in kinetic mechanisms or models.

Hence, the plots of substrate concentration versus the respective velocities obtained for Gln-AMC and Gln-βNA follow different kinetic laws. Whereas the kinetic data for Gln-AMC readily resembled Michaelis-Menten kinetics in the concentration range limited by substrate solubility, Gln-βNA showed discernible substrate inhibition (Figure 3). Interestingly, papaya QC showed a higher selectivity for Gln-βNA, but was inhibited by the substrate with similar potency. To our knowledge, Gln-βNA is the only QC substrate showing differences to Michaelis-Menten-kinetics, which could be indicative for a similar catalytic action of both enzymes.

Subsequently, the pH-dependence of the catalytic parameters $k_{\text{cat}}$ and $K_M$ for conversion of Gln-AMC by human QC was investigated. The pH-range used was restricted to 5.5-8.5, due to the limited stability of QC and the auxiliary enzyme at more basic and acidic pH. Similar experiments were already performed with papaya QC using Gln-tBE as substrate [17], revealing that the catalytic activity depends only on changes of substrate binding. Apparently, the substrate having a protonated amino group was not bound by the active site. The rate constant $k_{\text{cat}}$ did not change in
the investigated pH-range. Also human QC-catalysis exhibited only a dependence in terms of substrate binding, reflected by a pH-dependent change of $K_M$, and a pH-independent $k_{cat}$ (Figure 4). Fitting of the pH-dependent kinetic data of $K_M$ to an equation that accounts for two dissociating groups revealed a $pK_a$-value that is very close to the $pK_a$ of the substrate amino group and a second $pK_a$, probably representing a dissociating group of the enzyme. Thus, human and papaya QC bind only $N$-terminally unprotonated substrate molecules in a catalytically productive manner, indicating some general similarity in the catalytic mechanism, in spite of a lack of structural homology. Differences, however, where observed in the binding of inhibitory compounds. Whereas papaya QC was inhibited by peptides bearing $N$-terminal proline [12], human QC was not. We found, however, competitive inhibition of human QC by peptides bearing $N$-terminal $\gamma$-glutamyl-hydrazide residues (Figure 5). Furthermore, human QC was also inhibited by imidazole derivatives which contrasts with plant QC (not shown). These results suggest differences of plant and human QC concerning substrate conversion, apparently due to differences in the substrate and inhibitor recognition modes. Although the described assays have shown to be suitable for many applications, there is one major disadvantage. Only QC substrates can be used, whose conversion yields finally chromophoric or fluorogenic groups. Thus, the substrate spectrum is limited to variations of the chromophores and fluorophores, which hampers a detailed substrate specificity investigation. Therefore, alternative assays were needed, that overcome this drawback without waiving a continuous data monitoring.

**Coupling QC to glutamic dehydrogenase (GDH) catalysis – development and application of an alternative assay.** This assay is based on quantification of ammonia, that is liberated by cyclisation of glutamine. The auxiliary enzyme in this assay is glutamic dehydrogenase, converting ammonia, $\alpha$-ketoglutaric acid and NADH/H$^+$ into glutamic acid and NAD$^+$. Since the absorbance characteristics of NADH/H$^+$ changes by oxidation, its conversion can be followed at 340 nm. QC activity can be subsequently quantified by calculation of the liberated ammonia from standard curves of ammonia under assay conditions. The assay reactions are illustrated in Figure 6.
Originally, the assay was developed as a discontinuous method [16], probably due to the relatively low affinity of GDH towards ammonia. In turn, this low reactivity leads to very high auxiliary enzyme concentrations necessary to implement conditions that enable a continuous data monitoring according to equation 3. The usage of common cuvettes for spectrophotometers requiring sample volumes of 1-2 ml, however, causes the consumption of tremendous amounts of GDH, making the assay prohibitive. A calculation of the required auxiliary enzyme amount (equation 3) resulted in 30 U/ml GDH needed to reach a virtual steady state 20 s after initiation of the reaction (assuming a K_M of 3.2 mM of GDH for ammonia [24] and that one Unit of GDH refers to saturating concentrations of all substrates). Due to implementation of microplate readers for assay development, it was possible to reduce the assay volume to 250 µl, thus keeping the required auxiliary enzyme amount low, but still providing convenient volumes for pipetting. Furthermore, the use of the microplates, that were already applied in the fluorometric assays, enable a fast determination of QC activity in many samples at the same time, thus accelerating the determinations enormously. Finally, due to the detection of ammonia that is liberated from the QC-substrates the assay can be implemented for a fast examination of a variety of glutaminyl-peptides.

In fact, linear progress curves were obtained according to the predicted conditions, and most importantly there was a linear relationship between QC-concentration and initial velocity, indicating that the assay provides reliable results (Figure 7). Subsequently, a detailed substrate specificity investigation was performed using about 40 newly synthesised substrates (Schilling et al., submitted), showing that the assay is applicable independently from changes of substrate amino acid composition and peptide size. Moreover, since the auxiliary enzyme was not influenced significantly by potassium chloride concentrations up to 0.5 M, the method was also implemented to investigate the influence of ionic strength on conversion of different substrates. Interestingly, the observed changes of the specificity constant k_cat/K_M were substrate dependent (Figure 8). Neither papaya QC, nor human QC revealed altered activity by increasing ionic strength towards peptides with uncharged backbone and residues. Both enzymes, however, displayed a significant increase in
activity towards peptides comprising positively charged amino acid residues. Thus, besides the similar pH-dependence of catalysis for both enzymes, also the behaviour of the different QC-forms in environments with differing dielectric constants is similar, which could be also indicative for analogous catalytic mechanisms. Finally, this conclusion was also corroborated by the ability of both, human and papaya QC, to cyclise $N$-terminal $\beta$-homoglutaminyl residues with the same catalytic efficiency (not shown).

**Conclusions**

The application of theoretical deductions [18] facilitated the development of the first coupled enzymatic assays for Glutaminyl cyclase (QC) activity. Due to the use of different enzymatic reactions for coupling to QC catalysis, many characterisation studies could be performed including pH-dependence, inhibitory and substrate specificity. In this regard, the general differences in the coupling strategy, i.e. the consumption of the QC-products, either the pyroglutamyl peptide by pGAP or the liberated ammonia by GDH, led to a compensation of respective disadvantages of both assays. For instance, traces of ammonia in a sample hamper the QC-determination in the GDH-coupled assay, but show no effect in the assays using pGAP, resulting in the preferred usage of the latter enzyme for assays during enzyme purification. When investigating different peptide substrates, however, only the coupling to ammonia production provided satisfying results since a large substrate spectrum that can be investigated. Thus, the use of the different assay coupling strategies enabled the convenient determination of QC activity in different fields of protein characterisation.

Finally, the demonstrated strategy to develop continuous assay techniques could also be used to modify discontinuous assays for other enzymes or to develop new ways for their catalytic characterisation by implementing different coupling strategies.
Acknowledgement

We thank K. Zunkel, H. Cynis and J. Zwanzig for valuable technical assistance.
References


Table 1: Times to reach virtual steady state conditions in QC assay by coupling to pyroglutamyl aminopeptidase (1 U/ml), calculated according to equation 3. The kinetic data for pGAP were obtained from references [20] and [21]a.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_B$ (mM)</th>
<th>$V_{max}^2$ (µmol mg⁻¹ min⁻¹)</th>
<th>time to reach 95% $[B_{ss}]$ in QC assay at 1 U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGlu-pNA</td>
<td>0.69</td>
<td>0.56</td>
<td>2 min</td>
</tr>
<tr>
<td>pGlu-AMC</td>
<td>0.33</td>
<td>5.7</td>
<td>6 s</td>
</tr>
<tr>
<td>pGlu-βNA</td>
<td>0.13</td>
<td>20.0</td>
<td>&lt; 1 s</td>
</tr>
</tbody>
</table>
**Figure captions**

**Figure 1**: Representation of the coupled QC-assay using Gln-βNA as substrate. In the initial reaction, Gln-βNA is converted by QC into pGlu-βNA. Subsequently, the intermediate is cleaved by the abundant pyroglutamyl aminopeptidase into pyroglutamic acid and the fluorophore 2-naphthylamine, resulting in an increase in the observed fluorescence.

**Figure 2**: Progress curves of the conversion of Gln-βNA and Gln-AMC by QC, investigated by coupling the reaction to pGAP catalysis. According to a calculation (Equation 3), linear progress curves were observed directly after initiation of the reaction (Gln-βNA and Gln-AMC). Assays were carried out in 0.05 M Tris/HCl, pH 8.0 at 30 °C. The substrate and QC concentrations were 0.25 mM and 0.9 nM, respectively.

**Figure 3**: Dependence of the conversion-rate of Gln-βNA from the substrate concentration, determined for human (α) and papaya QC (B). The resulting graphs were obtained by fitting the data to the general equation of substrate inhibition. Human QC (K_M = 70 ±3 µM, k_cat = 21 ±1 s^{-1}, K_i = 1.21 ±0.07 mM) showed a reduced specificity compared to papaya QC (K_M = 36 ±2 µM, k_cat = 49 ±1 s^{-1}, K_i = 1.14 ±0.05 mM), but the catalysis was inhibited to similar extends by the substrate. Reactions were carried out in 0.05 M Tris/HCl, pH 8.0 (human QC) or 0.05 M Tricine/NaOH, pH 8.0 (papaya QC) at 30 °C.

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*The unit definition refers to pGlu-pNA. One unit of pGAP is defined as 1 µmol substrate converted per min under the described conditions.*
**Figure 4:** The pH-dependence of Gln-AMC conversion by human QC. At varying pH-values, the kinetic parameters $K_M$ and $k_{cat}$ were determined, and the logarithmic values plotted. Whereas the $k_{cat}$-value ($B$) was independent from pH, the $K_M$-values ($\alpha$) increased in the acidic and basic pH-region. Fitting the data to an equation that accounts for two dissociating groups resulted in pKa-values of $6.81 \pm 0.04$ and $8.6 \pm 0.1$. The former value is in good agreement with the pKa of the substrate whereas the latter probably reflects a dissociating group of the enzyme. Reactions were carried out in a buffer system providing a constant ionic strength over the entire pH-range, consisting of 0.06 M acetic acid, 0.06 M Mes and 0.12 M Tris [25] at 30 °C.

**Figure 5:** The array of curves obtained for the conversion of Gln-AMC by human QC in presence of varying concentrations of the inhibitory active peptide H-Glu(NH-NH$_2$)-Ser-Pro-Thr-Ala-NH$_2$. Data points were fitted according to the general equation for competitive inhibition. The resulting $K_i$-value was $0.697 \pm 0.003$ mM. The assay was carried out in 0.05 M Tris/HCl, pH 8.0, containing 5 mM EDTA. The substrate concentrations ranged from 1 mM to 0.125 mM.

**Figure 6:** Representation of the QC-assay using GDH as auxiliary enzyme and an N-terminal Glutaminyl peptide as substrate. In the initial reaction, the respective pyroglutamyl peptide and ammonia are formed. Subsequently, ammonia, $\alpha$-ketoglutaric acid and NADH/H$^+$ are converted into glutamic acid and NAD$^+$ catalysed by GDH. The consumption of NADH/H$^+$ can be observed at 340 nm.

**Figure 7:** Linear dependence of the initial rate of conversion on concentration of human QC using GDH as auxiliary enzyme. The inset shows two progress curves, in the sample containing human QC (12 nM), a linear decrease of absorbance was observed. Without added QC, the decrease in absorbance was negligible. Reactions were carried out in 0.05 M Tris/HCl pH 8.0, containing 5 mM EDTA.

**Figure 8:** The influence of ionic strength on the specificity constant $k_{cat}/K_M$ for conversion of
various substrates by human and papaya QC. For most peptides, there was little effect of changes in ionic strength detected. However, human and papaya QC specificity towards positively charged peptides increased significantly by addition of 0.5 M KCl. Without additional salt added, the ionic strength was 0.029 M, corresponding to a 0.05 M Tris- or Tricine buffer.

Figure 1:
Figure 2:
Figure 4:
Figure 5:
Figure 6:

\[ \text{NH}_3 + \text{NADH/H}^+ + \alpha\text{-ketoglutaric acid} \xrightarrow{\text{GDH}} \text{NAD}^+ + \text{glutamic acid} \]

\[ \text{H}_2\text{N} - \text{H} - \text{N} - \text{NH}_2 \rightarrow \text{QC} \rightarrow \text{peptide} \]

\[ \text{NH}_3 + \text{NADH/H}^+ + \alpha\text{-ketoglutaric acid} \xrightarrow{\text{GDH}} \text{NAD}^+ + \text{glutamic acid} \]
Figure 7:
Figure 8:
Inhibition of glutaminyl cyclase prevents the formation of pGlu³-amyloid-β related peptides

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Running Title: Discovering the glutamate cyclase activity of glutaminyl cyclase

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**Abbreviations:** AD, Alzheimer’s disease; APP, amyloid precursor protein; BACE, βNA, 2-naphthylamine; pGlu, pyroglutamic acid; QC, glutaminyl cyclase; EC, glutamyl cyclase; DP IV, dipeptidyl peptidase IV; Aβ(3-11)a, Amyloid β-peptide 3-11 amide, GnRH, Gonadotropin releasing-hormone; TRH, thyrotropin releasing-hormone
Abstract

N-terminal pyroglutamate (pGlu) has been identified as one of the major amyloid-β (Aβ) peptide components of plaques found in the brains of Alzheimer’s disease (AD) patients. Several related Aβ peptides contain N-terminal glutamate residues at positions 3 and 11. In parallel or subsequent to posttranslational β- and γ-secretase cleavage of the Aβ precursor protein (APP), N-terminal processing generates pGlu-Aβ peptides, molecular species are much more resistant to further degradation by aminopeptidases. Such N-terminal pGlu-peptide formation can be facilitated by glutaminyl cyclase (QC), a metal-dependent enzyme abundant in the brain and thought to be responsible for the ultimate processing of bioactive neuropeptides such as TRH and neurotensin during their maturation in the secretory pathway.

To clarify whether QC can also recognize Aβ-related peptides, the turnover of Gln³-Aβ(1-11)a, Aβ(3-11)a, Gln³-Aβ(3-11)a, Aβ(3-21)a, Gln³-Aβ(3-21)a and Gln³-Aβ(3-40) by the enzyme was investigated. Unexpectedly, we found that recombinant human QC as well as QC-activity from procine brain extracts catalyze both the N-terminal glutaminyl as well as glutamate cyclization. Most striking was the finding that cyclase-catalyzed Glu¹-conversion is favored around pH 6.0 while Gln¹-conversion to pGlu-derivatives occurs with a pH-optimum of around 8.0. Since the formation of pGlu-Aβ-related peptides can be suppressed by inhibition of recombinant human QC and QC-activity from porcine pituitary extracts, the enzyme QC is suggested as a novel target in drug development for AD-treatment.
Introduction

Regulatory peptides such as GnRH, TRH and neurotensin, and the cytokines MCP-1 through 4, require \textit{N}-terminal pyroglutamate in order to exert their respective biological functions (1;2). Early studies have suggested that the formation of pyroglutamate at the \textit{N}-terminus of glutaminyl peptides was a spontaneous reaction (3). Since this intramolecular reaction occurs only very slowly under physiological conditions, glutaminyl cyclase (QC; EC 2.3.2.5) was postulated and subsequently identified to be the catalyst responsible for the transformation of \textit{N}-terminal glutamine residues during posttranslational maturation of peptides in the secretory pathway of mammals and plants (Scheme 1) (4-7).

The first QC was isolated by Messer from the latex of the tropical plant \textit{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\textsuperscript{1}-precursors of TRH and GnRH into the appropriate mature pGlu\textsuperscript{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).

Coincidently, 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 amyloidotic plaques closely associated with dystrophic neurons, reactive astrocytes and microglia (10-16). Amyloid-\(\beta\) peptides are generated by proteolytic processing of the \(\beta\)-amyloid precursor protein (APP), which is cleaved \textit{N}-terminally by \(\beta\)-secretase (BACE) and \textit{C}-terminally by \(\gamma\)-secretase in a subsequent step (17-19) (scheme 2). It should be mentioned that there is much controversy concerning the ultimate involvement of the \(\gamma\)-
secretes activity of the presenilins in the formation of Aβ(1-40/42) and their utility as targets in AD-therapy (20;21).

Within the widely heterogeneous N-terminus of the amyloid peptide found in senile plaques exists a dominant fraction of Aβ-peptides containing an amino terminal aspartate residue such as Asp¹-Aβ(1-40) and Asp¹-Aβ(1-42). These full-length Aβ-peptides are found predominantly in the plaque periphery. In contrast, a second dominant fraction of Aβ-peptides, those with an N-terminal pyroglutamine, e.g. pGlu³-Aβ(3-40/42) and pGlu¹¹-Aβ(11-40/42), can be found preferentially in the core of senile plaques. These shortened peptides are reported to be more neurotoxic in vitro and to aggregate more rapidly than the full-length isoforms (9;22). N-terminally truncated peptides have been shown to be overproduced in early onset familial AD (FAD) subjects (23), to be dominant in diffuse plaques in the brains of patients with Down’s syndrome (DS) and AD (24), and to appear early and increase with age in Down’s syndrome brains (25-28). Further, their quantity has been shown to correlate with disease severity (25).

Among all prominent Aβ peptides, the isoforms containing pyroglutamate at position 3, such as pGlu³-Aβ(3-40/42), represent the most abundant of the N-terminally truncated peptide species (~ 50 % of total Aβ protein), particularly in the core of senile plaques (29-31). The accumulation of pGlu-Aβ peptides is likely due to the structural modification that enhances aggregation and confers resistance to most aminopeptidases (23;32). This evidence provides clues for a pivotal role of pGlu-Aβ peptides in AD pathogenesis. Cyclization, which yields the pyroglutamate form of an amyloidogenic peptide (with an uncharged N-terminus), may contribute to the overall hydrophobicity of the structure (9).

There are four potential pathways, which could lead to such neurotoxic pGlu-compounds:
(i) spontaneous cyclization of N-terminal glutamate residues following exposure by BACE and/or aminopeptidases

(ii) Glu to Gln mutations and/or posttranslational esterification or amidation of Aβ-glutamates buried within the APP-chain and subsequent spontaneous cyclization of the glutamines after N-terminal exposure by BACE and/or aminopeptidase processing

(iii) enzymatic cyclization of N-terminally uncovered glutamate

(iv) Glu to Gln mutations and/or posttranslational amidation of the Aβ-glutamates buried within the APP-chain and enzymatic cyclization after N-terminal exposure by BACE and/or aminopeptidase processing.

So far, there is no experimental evidence that is supportive of pathways (i) and (ii). The enzymatic conversion of Glu1-peptides into pGlu1-peptides by an unknown glutamyl cyclase (EC) corresponding to pathway (iii) was recently proposed (30). However, to date, no such enzyme activity has been identified, capable of cyclizing Glu1-peptides which are N-terminally protonated and possess a negatively charged Glu1 γ-carboxylate moiety. Hence, the remaining postulated path to N-terminal pGlu formation (iv) may involve glutaminyl cyclase activity as speculated previously (33). However, QC-activity against Gln1-substrates is dramatically reduced below pH 7.0 (Schilling et. al., 2003a1). Interestingly, Glu1-conversion has been reported to occur at acidic reaction conditions (24-30).

In order to prove whether QC is able to recognize and to turnover amyloid-β derived peptides under mild acidic conditions, we synthesized and investigated Gln3-Aβ(1-11)a, Aβ(3-11)a, Gln3-Aβ(3-11)a, Aβ(3-21)a, Gln3-Aβ(3-21)a and Gln3-Aβ(3-40) as potential substrates of the glutaminyl cyclases.

enzyme. These sequences were designed according to the sequences of naturally occurring N-terminally and C-terminally truncated Glu$^3$-Aβ peptides and Gln$^3$-Aβ peptides which could occur due to posttranslational Glu-amidation.

Another objective of the study was to compare the fate of the amyloid-β derived peptides using either porcine pituitary homogenate as source of native QC, purified recombinant human QC alone, or QC in combination with an aminopeptidase, under the rationale that aminopeptidase cleavage of full length Aβ peptide is a pre-requisite to QC cyclization of Glu/Gln at position three from the N-terminus. Finally, it was of interest whether human QC-activity processing of the above amyloid-β peptides can be suppressed by recently characterized QC-inhibitors (Schilling et al., 2003b$^2$).

**Experimental Procedures**

*QC Isolation*

Human QC was expressed in *P. pastoris* or *E. coli* and purified as described (34). Papaya QC was purified from papaya latex essentially as described elsewhere (35) with the addition of a third chromatography step on an UNO S (BioRad) column.

*Oligopeptide synthesis*

Amyloid-β 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 (34) or using an automated Symphony peptide synthesizer (Rainin Instrument Co.) in a 50µmol scale. Amyloid-β peptide Gln$^3$-Aβ(3-40) was synthesized in 25 µmol scale on Fmoc-Val-

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NovaSyn® TGA resin (0.15 mmol/g) or on the NovaSyn® TGR resin (0.23 mmol/g) using the automated Symphony peptide synthesizer. For all peptide couplings modified Fmoc-protocols of solid-phase peptide synthesis were employed using 2-(1H-Benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium tetrafluoroborate (TBTU; Novabiochem)/\textit{N}-methyl-morpholine; (NMM; Merck) as coupling regents. The coupling reaction was carried out by using 5 eq of Fmoc-amino acid, 5 eq TBTU and 10 eq NMM employing double coupling (twice 30 min and twice 1h from the 21st coupling onwards). Deprotection was carried out by using 20% piperidine in DMF (twice 5 min, then from the 21st coupling step onwards, once 5 min and once 12 min).

After cleavage from the resin by a trifluoroacetic acid (TFA; Merck) containing cocktail, the crude peptides were purified by preparative HPLC with acid free solvents in order to avoid further cyclization of the \textit{N}-terminal glutamine. Preparative HPLC was performed with a gradient of acetonitrile in water (20 % to 65 % acetonitrile over 40 min) on a 250-10 Luna RP18, WP300 column (MERCK). To confirm peptide purity and identity analytical HPLC and ESI-MS were performed.

\textit{Assays of QC}

QC activity was evaluated fluorometrically using Gln-βNA at 30 °C, essentially as described (35). Glu-βNA was employed for observation of glutamate cyclization catalyzed by papaya QC. Spontaneous cyclization of Glu-βNA and Gln-βNA (2 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 fluorometrically analyzed (35).

For the pH-dependence studies under first-order rate-law conditions (i.e. substrate concentrations far below \textit{K}_{M}-values), a buffer was prepared consisting of 0.05 M acetic acid, 0.05 M pyrophosphoric acid and 0.05 M Tricine. The pH-value was adjusted by addition of NaOH. In order to compensate for differences in ionic strength between buffers, constant

conductivity was maintained by addition of NaCl to the different buffers prepared at different pH-values. The spectrophotometric assay of QC was applied as described elsewhere (Schilling et al., 2003a). Enzyme kinetic data was analyzed using Grafit software (version 5.0.4. for windows, Erithacus software Ltd., Horley, UK).

**MALDI-TOF mass spectrometry**

Matrix-assisted laser desorption/ionization mass spectrometry was carried out using a Hewlett-Packard G2025 LD-TOF System. Enzymatic reactions using the Gln-peptides were performed in samples of 100 µl consisting of QC (0.01- 1 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 further in the text. At the times indicated, samples were removed, diluted with matrix and analyzed as described previously (34).

For long-term testing of Glu¹-cyclization, Aβ-derived peptides were incubated in 100µl 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 hours. In the case of Aβ(3-21)a, the assays contained 1 % DMSO. At the times indicated in the Figures 1-3, samples were removed from the assay tube, peptides were extracted using ZipTips (Millipore) according to the manufacturer’s recommendations, mixed with matrix solution (1:1 v/v) and subjected to mass spectrometry. Negative controls did either contain no QC or heat deactivated enzyme. For the inhibitor studies the sample composition was the same as described above, with exception of the inhibitory compound added (5 mM benzimidazole or 2 mM 1,10-phenanthroline).

**Results**

*Synthesis of Aβ peptides*
The synthesis of Aβ(1-40/42) peptides is known to be difficult due to excessive hydrophobicity in the C-terminal region which leads to aggregation or secondary structure formation during stepwise solid-phase peptide synthesis. Several attempts have been made to overcome these difficulties for instance by using stronger coupling reagents such as HATU or more efficient base for deprotection like DBU (36;37). To increase coupling yields during synthesis of Gln\(^3\)-Aβ(3-40) we used a low pre-loaded resin (Fmoc-Val-NovaSyn\(^\text{®}\)TGA, 0.15 mmol/g). Furthermore, we introduced a pseudoproline unit (Fmoc-Gly-Ser(Ψ\(^\text{Me,Me}\)pro)-OH instead of Gly\(^{25}\)-Ser\(^{26}\) to disrupt aggregation of the peptide chain (38). The introduction of this pseudoproline unit resulted in a significant improvement in the purity and the yield of the crude amyloid peptide.

_Turnover of Gln\(^3\)-Aβ peptides 3-11a, 3-21a and 3-40 by recombinant human QC_

In previous work we characterized more than 30 Gln\(^1\)- and Glu\(^1\)-peptides of different chain lengths as potential substrates of glutaminyl cyclase (Schilling et. al., 2003a). While H-Gln-\(\text{βNA}\), H-Gln-Phe-Lys-Arg-Leu-NH\(_2\) and even H-\(\text{β-homoGln-Phe-Lys-Arg-Leu-Ala-NH}_2\) were recognized and \(N\)-terminally cyclized by human QC, compounds such as H-Glu(OMe)-Phe-Lys-Arg-Leu-Ala-NH\(_2\), H-Glu-\(\text{βNA}\) or H-Glu-Phe-Lys-Arg-Leu-Ala-NH\(_2\) neither served as substrates nor as inhibitors of the enzyme under the basic pH-conditions applied. However, Glu(OMe)-Phe-Lys-Arg-Leu-Ala-NH\(_2\) demonstrated a tendency towards spontaneous formation of pGlu-Phe-Lys-Arg-Leu-Ala-NH\(_2\). Consistent with these findings, Glu(OMe)\(^3\)-Aβ(3-11)a was also found to cyclize spontaneously (data not shown).

All Gln\(^3\)-Aβ derived peptides tested were efficiently converted by human QC into the corresponding pyroglutamyl forms (Table 1). Due to the poor solubility of Gln\(^3\)-Aβ(3-21)a and Gln\(^3\)-Aβ(3-40) in aqueous solution, the determinations were carried out in the presence of 1% DMSO. The higher solubility of Gln\(^3\)-Aβ(3-11)a, however, enabled kinetic analysis both
in the presence and absence of DMSO (Table 1). Taken together, the investigation of the Aβ peptides as QC-substrates with chain-length of 8, 18 and 37 amino acids (see Table 1) confirmed our previous observation that human QC-activity increases with the length of its substrates. Accordingly, Gln¹-gastrin, Gln¹-neurotensin, Gln¹-GnRH are among the best QC-substrates when taking the specificity constants into account (Schilling et. al., 2003a). Similarly, Gln³-Aβ(3-40) and glucagon, the largest QC-substrates investigated thus far, exhibited high second order rate constants (449 mM⁻¹s⁻¹ and 526 mM⁻¹s⁻¹ respectively) even in the presence of 1% DMSO (Table 1).

Interestingly, the kinetic parameters for the conversion of the investigated amyloid peptides did not change dramatically with increasing size, suggesting only moderate effects of the C-terminal part of Aβ on QC catalysis. Therefore, due to better solubility and experimental handling, the further investigations concerning N-terminal aminopeptidase processing of these peptides were performed using the smaller fragments of Aβ, Gln³-Aβ(1-11)a, Gln³-Aβ(3-11)a and Aβ(3-11)a.

*Processing of Gln³-Aβ(1-11)a by purified DP IV and QC present in porcine pituitary homogenate*

After β-secretase post-methionine cleavage at position 670 of APP, further N-terminal degradation of the resulting Aβ peptide(s) occurs until the decomposition is halted by formation of N-terminal pGlu *in vivo* (22-28). Such concerted posttranslational processing mediated by aminopeptidases, dipeptidyl peptidase IV (DP IV) and glutaminyl cyclase has been already proposed for the formation of mature neuropeptides (39). Since full length Aβ(1-42) starts with the dipeptide Asp-Ala (a DP IV-recognition sequence) before the Glu in position 3, we investigated whether purified DP IV 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 DP IV 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 Figure 1A, 1C).

When the reaction was conducted in the presence of the DP IV-inhibitor Val-Pyrr, no further turnover of Gln$^3$-Aβ(1-11)a was observed (Figure 1B). Similarly, in the presence of the QC-inhibitor 1,10-phenanthroline, no final N-terminal pGlu-formation occurred, resulting in build-up of Gln$^3$-Aβ(3-11) as the final reaction product (Figure 1D).

When Gln$^3$-Aβ(1-11)a was incubated with porcine pituitary homogenate in the absence of both inhibitors, a slow stepwise removal of both amino acids by brain aminopeptidases and final cyclization to pGlu$^3$-Aβ(3-11)a by porcine pituitary QC and by DP IV-containing pituitary homogenate takes place (Figure 2A).

In the presence of the QC-inhibitor however, only the slow processing by brain aminopeptidases can be observed, yielding the intermediates Gln$^3$-Aβ(2-11)a and Gln$^3$-Aβ(3-11)a (Figure 2B). By incubating Gln$^3$-Aβ(1-11)a with DP IV-containing porcine pituitary homogenate in the presence of the DP IV-inhibitor Val-Pyrr only the formation of Gln$^3$-Aβ(2-11)a was detectable (Figure 2C).

**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 in contrast to previous work, glutamate-containing peptides can also serve as QC-substrates (Figures 3C and D). The QC-catalyzed 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 the addition of QC, substrate conversion resulting in pGlu$^3$-Aβ(3-11)a or pGlu$^3$-Aβ(3-21)a was suppressed (Figures 3E and F). If QC was boiled before addition, formation of the pGlu-peptides was negligible (Figures 3A and B).
pH-dependency 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 a very similar substrate specificity pattern to human QC (Schilling et al., 2003a). Accordingly, plant QC cyclized Aβ(3-11)\(_a\) at pH 5.2 (not shown). In contrast to human QC, however, we also observed the conversion of the short fluorogenic substrate Glu-βNA, which enabled us to apply a continuous coupled fluorometric assay (35). Since a different impact of substrate protonation on QC-catalysis was expected, higher amounts of QC were applied in the model reactions (34;35). Papaya QC converted Glu-βNA in a concentration range up to 2 mM (which was limited by substrate solubility) in accordance with Michaelis-Menten kinetics (Figure 4). Inspection of turnover versus substrate concentration diagrams for the conversion of Glu-βNA between pH 6.1 and 8.5 revealed that both \(K_M\) and \(k_{cat}\) changed in a pH-dependent manner (Figure 4). This is in contrast to the previously described QC-catalyzed glutamine cyclization, for which only changes in \(K_M\) were observed over the given pH range (40).

Subsequently, to study the impact of the proton concentration during Glu- and Gln-cyclization by QC, we investigated the pH-dependence of cyclization of Glu-βNA and Gln-βNA under first-order rate-law conditions (i.e. substrate concentrations far below \(K_M\)-values) (Figure 5). As expected the cyclization of glutamine has a pH-optimum at pH 8.0, in contrast to the cyclization of glutamic acid which 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 EC activity around pH 6.0, is only about 8,000.

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 \(1.2 \times 10^{-7} \text{ s}^{-1}\). However, during the same time
period, no pGlu-βNA was formed from Glu-βNA enabling estimation of a limiting rate constant for turnover of 1.0*10⁻⁹ s⁻¹.

**Discussion**

Since spontaneous formation of pGlu³-Αβ(3-40) occurs neither *in vitro* nor *in vivo*, only enzymatic cyclization of Glu-Αβ by a putative glutamyl cyclase (EC) or of Gln-Αβ peptides by the known glutaminyl cyclase (QC) is conceivable. Here, we have shown that papaya and human QC catalyze both glutaminyl and glutamylation cyclization (Schemes 1 and 3, Figures 1-5). The subcellular localization of these reactions remains unclear. Neurons can maintain in the cytosol pH values between 5.5 and 7.2 and cytosolic proteins of nerve cell enzymes are optimized for function at acidic pHS (41-43). Hence, under mildly acidic conditions preferred QC-catalyzed EC-reactions as observed in our study can occur in the cytosol.

However, Glu-Αβ peptides have been found to be preferentially generated by β-secretase processing in the endopasmatic reticulum as Αβ(1-40/42) and in the trans-golgi network as Αβ(11-40/42) most likely within secretory vesicles (44;45). Interestingly, QC is also localized in the secretory pathway (4-7) and by coincidence the major pGlu-Αβ peptides pGlu³-Αβ(3-40/42) and pGlu¹¹-Αβ(11-40/42) have been found in senile plaques of aged and Down syndrome brains (46).

The primary physiological function of QC is likely terminal hormone processing (maturation) in endocrine cells by glutamine cyclization prior to, or during the hormone secretion process. Such secretory vesicles are known to be acidic in pH. Thus, an auxillary/additional function of the enzyme in the narrow pH-range from 5.0 to 7.0 could be its newly discovered glutamyl cyclase activity (Scheme 3) transforming Glu-Αβ peptides. However, due to the relatively inefficient rate of Glu-cyclization compared to Gln-conversion, it is questionable whether the
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.

Investigating the pH-dependency of this enzymatic reaction, we found that the unprotonated N-terminus was essential for the cyclization of Gln₁-peptides and accordingly that the pKₐ-value of the substrate was identical to the pKₐ-value for QC-catalysis (see Figure 5 and Schilling et. al., 2003a). These results support the view that QC stabilizes the intramolecular nucleophilic attack of the unprotonated α-amino moiety on the γ-carbonyl carbon electrophilically activated by amidation (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 pKₐ-values of about 4.2 and 7.5 for the γ-carboxylic and for 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 unprotonated, and so exercising no electrophilic carbonyl activity. Hence, intramolecular cyclization is impossible.

However, in the pH-range of about 5.2-6.5, between their respective pKₐ-values, the two functional groups are present both partially non-ionized, in concentrations of about 1-10% (-NH₂) or 10-1% (-COOH) of total N-terminal Glu-containing peptide. As a result, over a mildly acidic pH-range species of N-terminal Glu-peptides are present which carry both groups uncharged, and, therefore, it is possible that QC could stabilize the intermediate of intramolecular cyclization to pGlu-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. At this pH the hydroxyl ion functions as a leaving group (Scheme 3). 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 to around pH 6.0, i.e. the pH-range in which substrate molecule species are simultaneously abundant carrying a protonated γ-carboxyl and unprotonated α-amino group. Furthermore, the kinetically determined pKₐ-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).

Physiologically, at pH 6.0 the second-order rate constant (or specificity constant, kₐ/Kₘ) of the QC-catalyzed glutamate cyclization might be in the range of 8,000fold slower than the one for glutamine cyclization (compare data in Figure 4). However, the non-enzymatic turnover of both model substrates Glu-βNA and Gln-βNA is negligible, which corroborates with the observed negligible pGlu-peptide formation in our study. Hence, for the pGlu-formation by QC an acceleration of at least 10⁸ can be estimated from the ratio of the enzymatic versus non-enzymatic rate constants (comparing the second-order rate constants for the enzyme catalysis with the respective nonenzymatic cyclization first-order rate constants the catalytic proficiency factor is about 10⁹ and 10¹⁰ M⁻¹ for the Gln- and the Glu-conversion, respectively). The conclusion from these data is, that in vivo only an enzymatic path resulting pGlu-formations seems conceivable.

Since QC is highly abundant in the brain and taking into account the high turnover rate of 0.9 min⁻¹ recently found for the maturation of 30 µM of (Gln-)TRH-like peptide (47), one can predict a cyclization half-life of about 100 hours for an appropriate glutamate-substrate, providing similar reaction conditions. Moreover, given compartmentalization and localization of brain QC/EC in the secretory pathway, the actual in vivo enzyme and substrate concentrations and reaction conditions might be even more favorable for the enzymatic cyclization in the intact cell. In addition, if N-terminal Glu is transformed to Gln a much more
rapid pGlu-formation mediated by QC could be expected. In vitro, we were able to suppress both reactions by applying inhibitors of QC/EC-activity (Figures 2 and 3).

Whether N-terminal processing of the tested Aβ-derived peptides takes place in vivo by a combination of aminopeptidase, dipeptidyl peptidase and glutaminyl/glutamyl cyclase activity and whether its selective blockage as achieved in our experiments is of physiological relevance begs further investigation. Previously, such N-terminal processing in an analog fashion was suggested for the immature neuropeptide Antho-RFamide precursors of coelenterates (39). Similarly, impaired post-translational proteolytic processing of Aβ peptides by aminopeptidases has been suggested as a causative event in AD (48). An imbalance between anabolic and catabolic processes of the APP-biosynthesis and the Aβ peptide degradation could cause the accumulation of Aβ(1-42) and other Aβ-peptides which are normally further degraded. According to this hypothesis, such an imbalance would occur through a reduced aminopeptidase activity which would not sufficiently process the N-terminus of Aβ resulting in a negligible iso-Asp<sup>1</sup>-Aβ(1-42) formation as compared to a significant accumulation of pGlu-Aβ peptides (23-29;46).

Recent reviews on potential AD treatment do not propose inhibition of pyroglutamate formation as potential therapeutic intervention (9;17;18;48). Since pGlu-Aβ peptides appear highly abundant, because they are even more hydrophobic and neurotoxic than the N-terminally charged amyloid peptides and since pGlu-Aβ formation prevents intracellular aminopeptidase-mediated disposal of such improperly generated peptides (44;46), inhibition of brain glutaminyl cyclase QC- and EC-activity could prove a valuable tool to combat the onset and progression of neurodegenerative disorders. In addition, a combination of the inhibition of QC/EC and aminopeptidases might be a suitable experimental approach to further investigate the site of the plaque-formation resulting from pGlu-Aβ peptides in situ and in vivo.
In summary, our results indicate that human QC/EC, which is highly abundant in the brain, is a likely catalyst to the formation of the amyloidogenic pGlu-Aβ peptides (from Glu-Aβ and Gln-Aβ precursors), major constituents of the senile plaques found in AD. In addition to their contribution towards our basic understanding of peptide processing during hormone maturation, these findings identify QC/EC as a potential player in plaque formation and thus as a novel drug target in the treatment of AD.

Acknowledgement

We thank H.-H. Ludwig for excellent technical assistance, M. Wermann for providing a DP IV-sample and we are grateful to Dr. J.A. Pospisilík for critical comments on the manuscript.
References


## Tables

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

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_M$ (mM$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gln$^1$-A$\beta$(3-11)a</td>
<td>$87 \pm 3$&quot;</td>
<td>$55 \pm 1&quot;$</td>
<td>$632 \pm 10&quot;$</td>
</tr>
<tr>
<td>Gln$^3$-A$\beta$(3-11)a</td>
<td>$155 \pm 4$</td>
<td>$41.4 \pm 0.4$</td>
<td>$267 \pm 4$</td>
</tr>
<tr>
<td>Gln$^3$-A$\beta$(3-21)a</td>
<td>$162 \pm 12$</td>
<td>$62 \pm 3$</td>
<td>$383 \pm 10$</td>
</tr>
<tr>
<td>Gln$^3$-A$\beta$(3-40)</td>
<td>$89 \pm 10$</td>
<td>$40 \pm 2$</td>
<td>$449 \pm 28$</td>
</tr>
<tr>
<td>Glucagon(3-29)</td>
<td>$19 \pm 1$</td>
<td>$10.0 \pm 0.2$</td>
<td>$526 \pm 17$</td>
</tr>
</tbody>
</table>

" Determined in absence of DMSO
**Schemes**

**Scheme 1:** $N$-terminal cyclization of glutaminyl peptides by QC

![Diagram of Scheme 1: N-terminal cyclization of glutaminyl peptides by QC](image)

**Scheme 2:** Sequence fragment of human amyloid precursor protein (APP 770)

<table>
<thead>
<tr>
<th>Position</th>
<th>Amino Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>661</td>
<td>Ile Lys Thr Glu Glu Ile Ser Glu Val Lys Met $\beta$ Asp Ala Glu Phe</td>
</tr>
<tr>
<td>676</td>
<td>Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys $\alpha$ Leu Val Phe</td>
</tr>
<tr>
<td>691</td>
<td>Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile Gly Leu</td>
</tr>
<tr>
<td>706</td>
<td>Met Val Gly Gly Val Val Ile Ala $\gamma$ Thr Val Ile Val Ile Thr Leu</td>
</tr>
</tbody>
</table>

- Full length $\alpha\beta$ peptide sequence 1-42 corresponding to amino acids 672-713 of APP in black.
- $\alpha$, $\beta$, $\gamma$-secretase cleavage sites marked bold and underscored.
- Glu$^3$ and Glu$^{11}$ of the $\alpha\beta$ peptide marked bold and underscored.
- $N$-terminal aminopeptidase processing site Asp-Ala are in italics.

**Scheme 3:** $N$-terminal cyclization of uncharged glutamyl peptides by QC (EC)

![Diagram of Scheme 3: N-terminal cyclization of uncharged glutamyl peptides by QC (EC)](image)
**Figure Legends**

**Figure 1:**  
A Mass spectra of Gln$^3$-Aβ(1-11)a incubated with DP IV catalyzing the N-terminal truncation yielding Gln$^3$-Aβ(3-11)a.  
B Mass spectra of Gln$^3$-Aβ(1-11)a incubated with DP IV and the DP IV-inhibitor Val-Pyrrolidide (Val-Pyrr) 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.

**Figure 2:**  
A Mass spectra of Gln$^3$-Aβ(1-11)a incubated with DP IV-containing porcine pituitary homogenate resulting in the formation of pGlu$^3$-Aβ(3-11)a after consecutive catalysis by both enzymes.  
B Mass spectra of Gln$^3$-Aβ(1-11)a in the presence of DP IV-containing porcine pituitary homogenate and the QC-inhibitor 1,10-phenanthroline preventing pGlu$^3$-Aβ(3-11)a formation.  
C Mass spectra of Gln$^3$-Aβ(1-11)a in the presence of DP IV-containing porcine pituitary homogenate and the DP IV-inhibitor Val-Pyr suppressing the formation of pGlu$^3$-Aβ(3-11)a.

**Figure 3:**  
A and 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 and 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 and 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.

**Figure 4:**  
Reaction rates of papaya QC- catalyzed Glu-βNA-conversion plotted against the substrate concentration. The initial rates were measured in 0.1 M pyrophosphate buffer, pH 6.1 (squares), 0.1 M phosphate buffer, pH 7.5 (circles) and 0.1 M borate buffer, pH 8.5 (triangles). The kinetic parameters were as follows: $K_M = 1.13 \pm 0.07 \text{ mM}$, $k_{cat} = 1.13 \pm 0.04 \text{ min}^{-1}$ (pH 6.1); $K_M = 1.45 \pm 0.03 \text{ mM}$, $k_{cat} = 0.92 \pm 0.01 \text{ min}^{-1}$ (pH 7.5); $K_M = 1.76 \pm 0.06 \text{ mM}$, $k_{cat} = 0.56 \pm 0.01 \text{ min}^{-1}$ (pH 8.5).
**Figure 5:** pH-dependence of the conversion of Gln-βNA (circles) and Glu-βNA (squares), determined under first-order rate-law conditions ($S \ll K_M$). Substrate concentration were 0.01 mM and 0.25 mM, respectively. For both determinations, a three-component buffer system was applied consisting of 0.05 M acetic acid, 0.05 M pyrophosphoric acid and 0.05 M Tricine. All buffers were adjusted to equal conductivity by addition of NaCl, in order to avoid differences in ionic strength. The data were fitted to equations that account for two dissociating groups revealing pKₐ-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 pKₐ-values of the respective substrate amino groups, determined by titration, were 6.97 ±0.01 (Gln-βNA) and 7.57 ±0.05 (Glu-βNA). All determinations were carried out at 30 °C.
Figure 1

A

B

C

D

800 1000 1200 1400 1600
mass/charge ratio (m/z)

Gln3-Aβ(1-11)α

Gln3-Aβ(3-11)α

pGlu3-Aβ(3-11)α

Gln3-Aβ(3-11)α

800 1000 1200 1400 1600
mass/charge ratio (m/z)

relative intensity

t= 1 min

t= 10 min

t= 30 min

t= 60 min

t= 90 min

t= 5 min

t= 10 min

t= 30 min

t= 60 min

t= 90 min
Figure 2

A

B

C
Figure 3

A

B

C

D

E

F

mass/charge ratio (m/z)

relative intensity

mass/charge ratio (m/z)

relative intensity

mass/charge ratio (m/z)

relative intensity

mass/charge ratio (m/z)

relative intensity

mass/charge ratio (m/z)

relative intensity

Figure 4

![Graph showing enzyme activity at different pH levels]
Figure 5

[Graph showing pH dependence of \( k_{cat}/K_M \) for Glu-\( \beta \)NA and Gln-\( \beta \)NA with data points and curves indicating the relationship between pH and enzymatic activity.]