Korean J Biol Sci 2: 243-248, 1998

Absence of an Essential Thiol in Human Glutaminyl Cyclase: Implications for Mechanism

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Key Words:
Glutaminyl cyclase
Cysteine residues
Chemical modification
Solvent isotope effect
Pyroglutamyl-peptide formation

We have partially sequenced glutaminyl cyclases from several mammalian and one avian species and found that the two cysteine residues of the human glutaminyl cyclase are completely conserved. The mammalian glutaminyl cyclase has been reported to possess reactive thiols (Busby, Jr, et al., 1987, *J Biol Chem* 262, 8532-8536). Mutagenesis of these cysteine residues, however, resulted in only a slight decrease in enzyme activity. Likewise, the recombinant human enzyme was completely resistant to attempted chemical modification of the putative reactive thiols. Although the human glutaminyl cyclase did not appear to have reactive thiols, it was sensitive to diethylpyrocarbonate and acetylimidazole, indicating the presence of functionally important histidine and tyrosine residues which could act as acid/base catalysts. Almost identical deuterium solvent isotope effect (1.2 vs 1.3) upon the reaction by the human and papaya enzymes, respectively, provides an evidence both animal and plant glutaminyl cyclases catalyze pyroglutamyl-peptide formation by intramolecular cyclization.

Glutaminyl cyclase (EC 2.3.2.5, glutamine cyclotransferase, QC) is an enzyme of the neuroendocrine system which catalyzes the cyclization of glutaminyl residues to pyroglutamyl residues at the amino terminus of numerous bioactive peptides. This enzyme was first reported as a glutamine cyclotransferase in papaya latex by Messer and Ottesen (1964, 1965) but recently the first detailed investigation into the catalytic mechanism of papaya QC has been published (Gololobov et al., 1994, 1996). The mammalian glutaminyl cyclase was reported independently in 1987 by the laboratories of Spiess (Fischer and Spiess, 1987) and Kizer (Busby, Jr, et al., 1987). Later Spiess and coworkers purified the bovine pituitary QC and obtained amino terminal peptide sequence, which enabled them to retrieve the cDNA sequence from a bovine pituitary cDNA library (Pohl et al., 1991). The sequence of the human pituitary enzyme later proved to be very similar to that of bovine QC, with 86% amino acid identity (Song et al., 1994). Among the invariant residues were two cysteines at positions 139 and 164. The initial reports by Kizer and coworkers using N-ethyl maleimide and immobilized mercurials (Busby, Jr, et al., 1987; Koger et al., 1989) suggested QC possessed at least one thiol required for enzymatic activity.

Our investigation of the papaya QC indicates the plant enzyme operates via a direct cyclization mechanism rather than the double displacement alternative which would involve a covalent acyl-enzyme intermediate. The presence of a reactive thiol in mammalian QC raises the possibility that it acts as a nucleophile during catalysis, and therefore animal QC operates by a different catalytic mechanism from the plant counterpart. To investigate this possibility, amino acid sequences of QC from several species of animals were examined for conservation of cysteine residues and the two cysteines present in human QC were mutated to examine the importance of their roles in the QC enzymatic reaction.

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Materials and Methods

Materials

Deuterium oxide, glutamate dehydrogenase, ampicillin, and chloramphenicol were from Sigma. *Taq* polymerase and restriction enzymes were from Promega, while *UlTma* DNA polymerase was from Perkin Elmer. RT-PCR First Strand Synthesis and PCR Optimizer kits were from Stratagene and Invitrogen, respectively. The Altered Sites Mutagenesis kit, utilizing the pALTER vector, was purchased from Promega. Bacterial expression vectors pET-19b and pGEX-4T-2 were from Novagen and Pharmacia, respectively. Expression and purification modules for the pGEX-4T-2 system were also obtained from Pharmacia, while Talon metal affinity resin was used for purification of proteins from the pET-19b system and purchased through Clontech.

BL21(DE3) pLysS competent cells and pT7 sequencing vector were obtained from Novagen. TRIzol reagent for RNA isolation was purchased from Gibco-BRL. Isopropyl β-*D*-thiogalactopyranoside (IPTG) was purchased from Amresco. Glutamine-amide (Gln-NH_2) was from Bachem and Gln-Gln was from Schweitzerhall. If not otherwise stated, the chemicals were of analytical grade.

Methods

Enzyme and protein assay: Glutaminyl cyclase activity was determined using the spectrophotometric assay as previously described by Bateman (Bateman Jr., 1989). Briefly, the assay used glutamate dehydrogenase to detect ammonia produced during the cyclization of the glutaminyl substrate. The decrease in absorbance at 340 nm resulting from the conversion of NADH to NAD^+ is proportional to enzyme activity. Denaturing gel electrophoresis was performed using a mini-gel system and 12% Novex precast gels. Proteins were visualized using Coomassie blue staining. Western blotting was performed using standard protocols with anti-bovine QC antibodies as described previously (Song et al., 1994).

Deuterium solvent isotope effect determination: Kinetic assays to assess the effect of deuterium oxide on QC activity were performed as described previously (Gololobov et al., 1994, 1996) with the following modifications. The wavelength was changed from 220 to 210 nm to increase the sensitivity of the assay. At this wavelength the substrate/product difference extinction coefficient is 562 $M^{-1}cm^{-1}$. Two millimolar Gln-NH_2 (Km=7.2 mM) in 0.1 M pyrophosphate buffer at pH 7.2 was used and the cuvets were maintained at 37℃ using a Fisher Isotemp circulating water bath attached to a Hitachi U2000 spectrophotometer. Fifty microliters of enzyme were added to 750 µl substrate to initiate the reactions, which were followed for 300 seconds. Initial rates were used instead of progress curves and were calculated using linear regression. To prepare buffer in deuterium oxide, the buffer prepared in water was lyophilized to complete dryness and redissolved in D_2O before dissolution of solid substrate as described previously (Gololobov et al., 1994). Both papaya and human QC reactions were performed under the described conditions.

QC amino acid sequences: Total RNA was extracted from sheep and pig pituitaries and dog, rat, and chicken hypothalamus using TRIzol reagent. Ten micrograms of RNA were then used in first strand synthesis and the resulting cDNA used in PCR. Optimization of QC amplification was necessary for each species tested. Bovine QC primers published previously (Song et al., 1994) were used in a series of Touchdown PCR reactions with each containing 2.5 mM each dNTP, 200 ng each primer, 5 µl cDNA, and 5 U *Taq* DNA polymerase. *UlTma* DNA polymerase was used for amplification of the pig PCR product. Touchdown PCR protocols allowed for the annealing temperature, typically beginning at 60℃, to drop 1℃ per two cycles with a total change in temperature of 10℃. Ten rounds of PCR were performed at the lowest annealing temperature of each run. Ampification products were analyzed on 1% TBE agarose gels and products from sheep, dog, and rat were subcloned into the pT7 vector for sequencing. Amplified products from pig, chicken, sheep, and rat were sequenced directly. Two clones containing the sheep and dog PCR products were also sequenced. Sequencing was performed at the University of Florida Core Sequencing facility and in every case both strands of DNA were sequenced. Genbank accession numbers for PCR products are AF039438 (pig), AF039439 (chicken), AF039440 (dog), AF039441 (sheep), and AF039308 (rat). Genbank accession numbers for the human and bovine QC DNA sequences are X71125 and M80626, respectively.

QC bacterial expression and purification: Expression of human QC was achieved utilizing two bacterial expression systems. The first system, using the pET-19b expression vector, has been previously described (Song et al., 1994). Soluble enzyme from this system was used for mutagenesis work, although quantities of soluble protein were limited due to the bulk of the fusion protein being put into inclusion bodies. Two mutants were created from the pET-19b human QC construct. Briefly, the cDNA fragment to be mutated was subcloned into the pALTER vector. Mutagenesis was designed to replace Cys by Ala at positions 139 and 164 using two mutagenic oligonucleotides (5′-ATTTGGTCCTCGTGCGCCACTATGACTCCA-3′ and 5′-TTCAGCCGTTGCCGGCTGCAATGATGTT-3′). This also resulted in the creation of unique restriction enzyme recognition sites for *Fsp*I and *Nae*I, respectively. Correctly mutated QC cDNA fragments were subcloned back into the pET vector. Two single mutants, Mut I (Cys139Ala) and Mut II (Cys164Ala), were created and identified by digestion with either *Fsp*I or *Nae*I, respectively, as well as sequencing.

The second system, using the pGEX expression system, produced a much higher quantity of soluble protein. Protein from this system was used for isotope effect studies as well as chemical modification studies. The human cDNA clone for glutaminyl cyclase was subcloned into a unique *Xho*I site in the pGEX-4T-2 vector, and the orientation was confirmed by restriction digestion and complete sequencing. The correctly oriented plasmid was then transformed into BL21(DE3) pLysS competent cells. Expression by standard procedures and purification over glutathione sepharose yielded the expected 67 kDa fusion protein of QC and *Schistosoma japonicum* glutathione S-transferase (GST) that

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COW       LLIERYPGSPGSFAARQHIMQRIQRLQADWVLEVDTFLSQTPYGYRSFSN  122
SHEEP_Q   LLIERYPGSPGSFAARQHIMQRIQRLQADWVLEVDTFLSQTPYGYRSFSN  122
PIG       LLIERYPGSPGSYAARQHIMQRIQRLQADWVLEIDTFLSQTPYGYRSFSN  122
DOG       LLIERYPGSPGSYAARQHIMQRIQRLQADWVLEIDTFLSQTPYGYRSFSN  122
CHICKEN     IERYPGSPGSYAARQHIMQRIQRLQADWVLEIDTFLSQTPYGYRSFSN  122
RAT       LLIERYPGSPGSYAARQHIMQRIQRLQADWVLEIDTFLSQTPYGYRSFSS  122
CONSENSUS LLIERYPGSPGSYAARQHIMQRIQRLQADWVLEIDTFLSQTPYGYRSFSN  122
          ********** ********************** ********************
                    CCCCCCCCCCCCHHHHHHHHHHHHHHHHHHHHHHEEECCCCCCCCCCCCCCCC

HUMAN     IIS7LNPTAKRHLVLACHYDSKYFSHWNNRVFVGATDSAVPCAMMLELAR  172
COW       IIS7LNPTAKRHLVLACHYDSKYFPHWDDRVFVGATDSAVPCAMMLELAR  172
SHEEP     IIS7LNPTAKRHLVLACHYDSKYFPHWDHRVFVGATDSAVPCAMMLELAR  172
PIG       IIS7LNPTAKRHLVLACHYDSKYFHPWDNRVFVGATDSAVPCAMLLELAR  172
DOG       IIS7LNPTAKRHLVLACHYDSKYFHPWDNRVFVGATDSAVPCAMLLELAR  172
CHICKEN   IIS7LNPTAKRHLVLACHYDSKYFHPWDNRVFVGATDSAVPCAMLLELAR  172
RAT       IIS7LNPTAKRHLVLACHYDSKYFHPWDNRVFVGATDSAVPCAMLLELXP  172
CONSENSUS IIS7LNPTAKRHLVLACHYDSKYFXXWDXRVFVGATDSAVPCAMXLELAR  172
          ********************** * ************** *** ***
                    CCCCCCCCHHHHHHEEECCCCCCCCCCCCCEEEECCCCCCCHHHHHHHHHHH

HUMAN     ALDKKLLSLKTVSDSKPDLSLQLIFFDGEEAFLHWSPQDSLYGSRHLAAK  222
COW       ALDKQLFSLKnISDSRPDLSLQLIFFDGEEAFHLWSPQDSLYGSRHLASK  222
SHEEP     ALDKQLFSLKnISDSRPDLSLQLIFFDGEEAFHLWSPQDSLYGSRHLASK  222
PIG       ALDKQLLSLEKIPDSKPDLSLQLIFFDGEEALLHWSLRDSLYGSRHLAPK  222
DOG       ALDKQLLSLEKIPDSKPDLSLQLIFFDGEEALLHWSLRDSLYGSRHLAPK  222
CHICKEN   ALDKQLLSLEKIPDSKPDLSLQLIFFDGEEALLHWSLRDSLYGSRHLAPK  222
RAT       SLDKQLLSLEKIPDSKPDPSLQLIFFDGEEALLHWSLRDSLYGSRHLAPK  222
CONSENSUS ALDKQLLSLXXIXDSKPDLSLQLIFFDGEEAXXHWSXXDSLYGSRHLAXK  222
          *** * **  ** ** ************  **  ********** * *
                    HHHHHHHHHHCCCCCCCCCCEEEECCCHHHHHHHHCCCCCCCCHHHHHHH

HUMAN     MASTPHPPGARGTSQLHGMDLLVLLDLIGAPNPTFPNFFPNSARWFERLQ  272
COW       MASTPHPPGARDTNQLHGMDLLVLLDLIGAPFPTFPNFFPNTARWFGRLE  272
SHEEP     MASTPHPPGARDTNQLHGMDLLVLLDLIGAPYPTFPNFFPNTARWFGRLE  272
PIG       MASTPHPPGAKDTNQLHGMDLLVLLDLIGAPNPTFPNFFPKSARWFNRLE  272
DOG       MASTPHPPGAKDTNQLHGMDLLVLLDLIGAPNPTFPNFFPKSARWFNRLE  272
CHICKEN   MASTPHPPGAKDTNQLHGMDLLVLLDLIGAPNPTFPNFFPKSARWFNRLE  272
RAT       MASTPHPPGAKDTNQLHGMDLLVLLDLIGAPNPTFPNFFPKSARWFNRLE  272
CONSENSUS MASTPHPPGAXDTNQLHGMDLLVLLDLIGAPXPTFPNFFPXSARWFXRLE  272
          ********** * ****************** ******** **** **
                    CCCCCCCCCCCCHHHHHHHHHHHHHHHHHCCCCCCCCCCCCCCHHHHHHHHH

HUMAN     AIEHELHELGLLKDHSLEGRYFQnYSYGGVIQDDHIPFL  311
COW       AIEHGLRELGLLKDHSSERWYFRNYGYGGVIQDDHIPFL  311
SHEEP     AIEHGLHELGLLKDHSSERRYFRNYGYGGVIQDDHIPFL  311
PIG       AIEQELHKLGLLKDYSLERCYFQnHSYGGVIQDD       311
DOG       AIEQELHKLGLLKDYSLERCYFQnHSYGGVIQDDHIPFL  311
CHICKEN   AIEQELHKLGLLKDYSLERCYFQnHSYGGVIQDDHIPFL  311
RAT       AIEQELHKLGLLKDHSLERCYFQnHSYGGAIQDDHIPFL  311
CONSENSUS AIEXELHXLGLLKDXSLERCYFXNXSYGGVIQDDHIPFL  311
          *** * ****** * *  ** * *** *********
                    HHHHHHHHHHHHCCCHHHHHHCCCCCCCCCCCCCCCC
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Fig. 1. Multiple sequence alignment of animal glutaminyl cyclases. Predicted posttranslational modification sites are identified by the following: underlined amino acids are protein kinase C phosphorylation sites, bold amino acids are casein kinase II phosphorylation sites, italicized amino acids are O-linked glycosylation sites, and lower case amino acids are N-linked glycosylation sites. Astericks are placed beneath residues identical throughout the alignment. Consensus secondary structure is given in the last row. Amino acids are numbered with respect to the start codon of human glutaminyl cyclase.

was immunoreactive with anti-QC antibodies.

Multiple alignment of mammalian PCR fragments: Multiple alignment, prediction of secondary structure, and possible posttranslational modification sites were determined using the program DSC: Discrimination of Protein Secondary Structure Class found at the Biomolecular Modelling Laboratory, Protein Sequence Analysis server at Boston University, and NetOGlyc 2.0 Prediction Server at the Center for Biological Sequence Analysis. PC GENE was also used for determination of possible posttranslational modification sites.

Chemical modification studies: The following compounds were examined for their ability to inhibit human QC enzymatic activity: acetylimidazole (5 mM), DEPC (1 mM), iodoacetamide (1 mM), iodoacetic acid (1 mM), N-ethyl maleimide (NEM) (5 mM), and phenylglyoxal (5 mM). The coupled spectrophotometric assay mentioned above (Bateman Jr., 1989) was utilized with the following modification. The GST-QC (45 ml) which had been dialyzed against the indicated pH was incubated with the appropriate compound (5 ml of stock to give the indicated final concentration) for 1 h at room temperature prior to the initiation of the assay by addition of 100 µl of substrate cocktail. Separate controls containing water (acetylimidazole, iodoacetamide, iodoacetic acid, and phenylglyoxal) and ethanol (NEM and DEPC) rather than modification reagents were utilized and the percent activity remaining after the assay was compared to the appropriate control reactions.

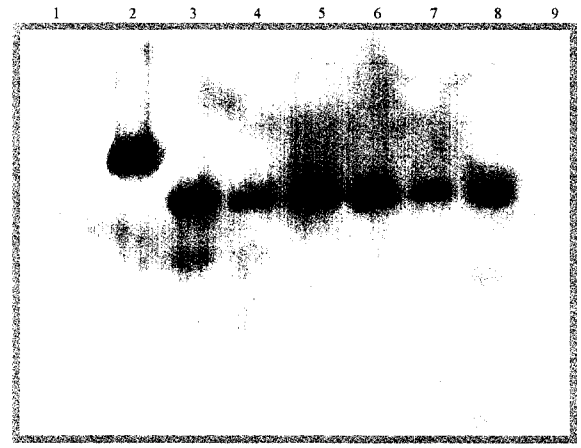

Fig. 2. Southern blot of animal glutaminyl cyclase PCR products. Lane 1, markers. Lane 2, Human QC clone (positive control). Lane 3, cow. Lane 4, sheep. Lane 5, pig. Lane 6, dog. Lane 7, chicken. Lane 8, rat. Lane 9, pGEM vector (negative control). Blot was probed with labeled human QC PCR product.

Results

Multiple QC amino acid sequence analysis

The cDNA sequences obtained by PCR were translated into amino acids and aligned (Fig. 1). Analysis of the amino acid sequences shows a remarkable degree of identity (81%) between the QC from mammalian species. To further confirm the high degree of identity among the species, a Southern blot was performed on all of the PCR products using a digoxigenin labeled human QC probe (Fig. 2).

Of particular importance is the conservation of the two cysteines previously reported by Pohl et al. (1991) and Song et al. (1994) in bovine and human QC, respectively, at positions 139 and 164 using the numbering system of the complete bovine sequence. It is also interesting to note the presence of a third cysteine residue at position 291 in chicken, rat, pig, and dog QC.

Nearly all of the potentially significant posttranslational modification sites were completely conserved among all species (Fig. 1). One O-linked glycosylation site at amino acid 126, three protein kinase C sites at positions 130, 208, and 264, and two casein kinase II sites at positions 187 and 208 were identified. There

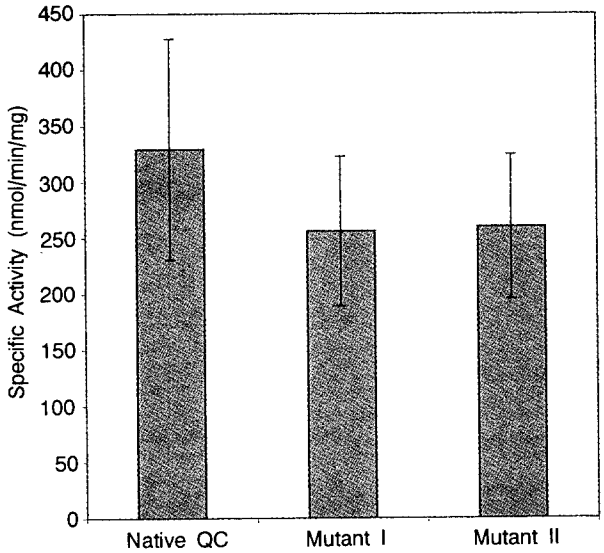

Fig. 3. Comparison of native glutaminyl cyclase and cysteine mutants. Mutant I is a Cys139Ala conversion and mutant II is a Cys164Ala conversion. Bars represent average of 9 determinations ± S.D.

were also two N-linked glycosylation sites conserved in some, but not all species, at positions 183 and 296. Analysis of both individual and multiply aligned sequences predicted the same secondary structure with no significant distinctions observed among the species.

QC mutant comparison

Native QC and mutants I and II were assayed using Gln-Gln as a substrate at a final concentration of 10 mM (Fig. 3). Native poly-His QC demonstrated a specific activity of 330 nmol/min/mg. Mutant I, which had an alanine replacing the cysteine at position 139, had a specific activity of 260 nmol/min/mg, 79% of the activity of the native enzyme. Mutant II, which had an alanine replacing the cysteine at position 164, had the same specific activity as mutant I.

GST-hQC expression

Expression of the recombinant pGEX-4T-2 vector produced a fusion product consisting of two proteins. Six amino acids bridged the two fusion domains encoding a thrombin cleavage site. To date, attempts to cleave the GST portion of the fusion protein were unsuccessful, suggesting a close association between the two domains. Sequencing of the pGEX construct region containing the human QC showed a single nucleotide change during cloning which resulted in a conservative Asn to Ser conversion at position 296. This is a relatively unconserved region of irregular secondary structure which is probably at the protein surface. Neither the GST fusion nor the mutation at position 296 appeared to affect enzyme activity.

Table 1. Chemical modification of recombinant human QC

Compound	Concentration	% Activity remaining
Acetyl imidazole	5 mM	44
Diethypyrocarbonate	1 mM	36
Iodoacetamide	1 mM	99
Iodoacetic acid	1 mM	100
N-ethylmaleimide	5 mM	98
Phenylglyoxal	5 mM	103

Chemical modifications

The results of the chemical modification studies of human QC are presented in Table 1. The sulfhydryl reagents, such as iodoacetamide, iodoacetic acid, and NEM, as well as the arginine-directed phenylglyoxal demonstrated no appreciable effect on the enzymatic activity of human QC. Preincubation with acetylimidazole and DEPC resulted in 45% and 64% losses in activity, respectively, which suggested the presence of functionally important tyrosine and histidine residues.

Solvent isotope effect

The deuterium solvent isotope effect for the recombinant human QC was calculated to be 1.20 ± 0.04, while the same effect on the purified papaya QC was 1.33 ± 0.02. Both were measured at pH 7.2 and were essentially identical to the previously reported isotope effect of 1.27 on the papaya enzyme at pH 8.8 (Gololobov et al., 1994).

Discussion

Despite the initial reports by Kizer and coworkers (Busby, Jr, et al., 1987; Koger et al., 1989) of a functionally important thiol group in porcine pituitary QC and the complete conservation of the two cysteines at positions 139 and 164 reported here, the removal of these thiols by mutagenesis appears to have very little effect on enzyme activity. This lack of an available thiol affecting enzyme activity was confirmed by testing a variety of chemical modification reagents against recombinant human pituitary QC. In particular, NEM was tested at concentrations up to 5 mM and at several pH values with no apparent effect. It is possible that the two conserved cysteine residues are disulfide-bonded, as are all the cysteines in other processing enzymes of the secretory pathway, such as peptidylglycine alpha-amidating monooxygenase (PAM) and dopamine β-hydroxylase (DBH) (Kolhekar et al., 1997), and even so the lack of the disulfide bond in the QC mutants did not appear to have a dramatic effect on either enzyme activity or observed stability under assay or storage conditions.

It is clear that there is little dependence of the purified recombinant human glutaminyl cyclase on a catalytic thiol. However, glutaminyl cyclase *in vivo* may be associated with a thiol-dependent second protein, possibly playing a regulatory or stabilizing role, which

was still present in the crude tissue extracts assayed by Kizer and coworkers. Such ancillary factors have been found for other secretory processing enzymes such as PAM (Eipper et al., 1991) and prohormone convertase 2 (Muller et al., 1997). The presence of high molecular weight forms of pituitary QC observed during gel filtration is consistent with this suggestion (Consalvo et al., 1988).

The high degree of amino acid identity makes elucidation of functionally important residues difficult to determine by simple sequence comparison. For example, inactivation with diethylpyrocarbonate points to a role for histidine residues in enzyme activity. There are seven histidines within the region shown in Fig. 1 which are completely conserved across all species. It is likely that one or more of these residues will be active site residues, but their identities will have to await further mutagenesis studies. Although PAM and DBH have clusters of essential histidine residues (Eipper et al., 1995; Yonekura et al., 1996), those histidines appear to be copper ligands rather than acid/base catalysts. Since QC is not a metalloprotein, it is unlikely that more than a few of the histidines will prove to be essential.

Little is known about the mechanism of action of mammalian glutaminyl cyclase. Other processing enzymes of the secretory pathway such as PAM and DBH have been studied extensively and a number of potent reversible and irreversible inhibitors of these enzymes have been published (Katopodis and May, 1990; Merkler et al., 1992, 1995; Rhodes and Honsinger, 1993; Eipper et al., 1995). Our previous kinetic and inhibitor studies of the papaya QC reaction mechanism point to a direct cyclization mechanism similar to that of gamma-glutamyl cyclotransferase rather than a covalent enzyme-intermediate complex such as that seen with gamma-glutamyl transpeptidase (Gololobov and Bateman, Jr, 1994a; Gololobov et al., 1994, 1996). Despite a complete lack of amino acid sequence similarity between animal and plant QC (Song and Bateman, unpublished observations), several lines of evidence indicate that both animal and plant QC operate by similar catalytic mechanisms. First, both enzymes are inhibited by compounds containing heterocyclic rings, with the inhibition of papaya QC by proline-containing peptides clearly acting as transition state analogs. Inhibition of pituitary QC by o-phenanthroline (Busby, Jr, et al., 1987; Consalvo et al., 1988) must be structurally based since QC is not a metalloenzyme. Second, the absence of either an essential thiol (this study) or a reactive hydroxyl group able to act as a nucleophile in human QC makes it difficult to conceive of a realistic covalent enzyme-intermediate mechanism (Busby, Jr, et al., 1987; Consalvo et al., 1988). Finally, the solvent isotope effect measured for the recombinant human QC is essentially identical to that of papaya QC (1.2 vs 1.3, respectively), consistent with their mechanistic similarity. Clearly the design of mechanism-based inhibitors of the known glutaminyl cyclases should be based on the premise that catalysis proceeds through a direct cyclization of the glutaminyl residue via a 5-membered cyclic intermediate, possibly involving histidine and tyrosine side chains as acid/base catalysts (Gololobov et al., 1994, 1996).

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HQCCOD  - MAG--GRHRRVVGTLHLLLLVAALPWASRGVSPSASAWPEEKNYHQPAIL  -48
YEASTQC - MGMKYVLPLRLIGLAYLLVLFQV---------HRVTGWELSYEQYHAA--  -39
HQCCOD  - NSSALRQIAEGTSISEMWQNDLQPLLI----ERYPGSPGSYAARQHIMQR  -94
YEASTQC - ------HLNEAINPDSGWNKSTKNLLLPFNRTRVPGSEGSREIQRFIIEH  -83
HQCCOD  - IQR-LQADWVLEIDTFLSQTPYGYRSFSNIISTLNPTAKRHLVLACHYDS  -143
YEASTQC - FNNTLAGEWAVETQAFEEN---GYR-FNNLVMTLQNNASEYLVLAAHYDT  -129
HQCCOD  - KYFSHWNNRVFVGATDSAVPCAMMLELARALDKKLLSLKT---------V  -184
YEASTQC - KI----APTGMVGAIDSAASCAALLYTAQFLTHIACHERTKEYNDLESNT  -175
HQCCOD  - SDSKPDLSLQLIFFDGEEAFLHWSPQDSLYGSRHLAAKMASTPHPPGARG  -234
YEASTQC - VVSNSTLGVKIVFFDGEEAIEEWGPEDSIYGARRLAAQWLADGTMTRIR-  -224
HQCCOD  - TSQLHGMDLLVLLDLIGAPN--PTFPNFFPNSARWFERLQAIEHELH---  -279
YEASTQC - ---------LLFLLDLLGSGEEEPLVPSYYAETHQEYQLLNRIEDDLLFRR  -266
HQCCOD  - ------ELGLLKDHSLEGRYFQNYSY------GGVIQDDHIPFLRRGVPV  -317
YEASTQC - GDEINGESALAAEVARQRKHLDPTDYRFLGLGHSVIGDDHTPFLAAGVPV  -316
HQCCOD  - LHLIPSPFPEVWHTMDDNEENLDESTIDNLNKILQVFVLEYLH---L    -361
YEASTQC - LHAIPLPFPSTWHTVDDDFRHLDAAETRHWALLVCEFVVQSLRSRNQ    -363
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Fig. 4. Alignment of human glutaminyl cyclase with putative yeast homolog. Comparison was made using the Dayhoff MDM-78 matrix with a resulting 32% identity and 11% similarity.

Although the tertiary structure of glutaminyl cyclase from any source has yet to be solved, the high degree of sequence identity between mammals and birds indicate a very similar folding pattern. It is tempting to speculate that the similarity in mechanism, substrate specificity (Gololobov et al., 1996), and size (~35 kD) between plant and animal QC may mean that this similarity at the tertiary structural level extends across the eukaryotic kingdoms. There is even an apparent QC homolog in yeast (Genbank accession number 1175991) having an overall identity of 32% with human QC and is only two amino acids longer (Fig. 4). Although this sequence has not yet been shown to represent a protein which contains the appropriate enzymatic activity, the sequence similarities to mammalian QC are striking. There are no known yeast proteins which contain the pyroglutamyl residue, but yeast do appear capable of forming this residue as evidenced by reports of successful heterologous expression of proteins containing the pyroglutamyl residue in *Saccharomyces* (Ito et al., 1992; Itoh et al., 1990; Sawai-Hatanaka et al., 1995). Of particular note in the context of this study is that the yeast protein contains three cysteine residues, only one of which corresponds to a cysteine (#164) in human QC. This suggests at least one of the cysteines examined in this study is not conserved through evolution, which would confirm the conclusion that cysteine 139 is not essential to the

enzymatic reaction. It will be interesting to note whether cysteine 164 continues to be conserved as new glutaminyl cyclase amino acid sequences are reported. If so, it may play a regulatory or transport role in the eukaryotic cell.

Acknowledgements

This work was supported by NIH Grant DK41892 to RC Bateman, Jr, the NSF EPSCOR Program, the state of Mississippi and the University of Southern Mississippi, and a research grant to I Song from the University of Seoul in 1996.

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[Received April 8, 1998; accepted May 6, 1998]