Evidence for Essential Histidines in Human Pituitary Glutaminyl Cyclase

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ABSTRACT: Glutaminyl cyclase (QC, EC 2.3.2.5) catalyzes the formation of the pyroglutamyl residue present at the amino terminus of numerous secretory peptides and proteins. Treatment with diethyl pyrocarbonate inactivated recombinant human QC with the apparent modification of three essential histidine residues. Comparisons of the protein sequences of QC from a variety of eukaryotic species show four completely conserved histidine residues. Mutation of each of these residues to glutamine resulted in two mutant enzymes that were inactive (H140Q and H330Q), suggesting a role in catalysis, and two that exhibited increased K_m values (H307Q and H319Q), suggesting a role in substrate binding. Consistent with these results is the prediction that QC possesses a zinc aminopeptidase domain in which the four histidines identified here are present in the active site. Mammalian glutaminyl cyclases may, therefore, have structural and catalytic similarities to a family of bacterial zinc aminopeptidases.

Many secretory peptides and proteins contain pyroglutamic acid (pGlu)[1] at the amino terminus. This posttranslational modification results from cyclization of a glutaminyl residue which is unmasked by proteolytic processing of precursors by prohormone convertases (*1*). These glutaminyl residues will slowly cyclize to the pyroglutamyl group in the absence of an enzyme catalyst, but the rate is greatly enhanced by glutaminyl cyclase (EC 2.3.2.5; QC), an enzyme originally reported in papaya latex in 1964 by Messer and Ottesen (*2*, *3*). To date, QC has been identified in a number of animals (*4*, *5*), plants (*6*), and bacteria (*7*). The human cDNA for QC has been previously cloned and expressed in a number of bacterial expression systems (*8*, *9*). There appears to be a single QC gene on human chromosome 2 (contig NT005367).

Initial studies of mammalian QC by Busby et al. (*4*) suggested the presence of reactive thiols. However, chemical modification studies and mutagenesis of both cysteine residues have indicated the lack of thiols essential for enzyme activity (*9*). Despite a complete lack of sequence homology between the plant and animal QC families (*6*), both enzymes have been presumed to proceed through acid/base promotion of the direct cyclization of the glutaminyl residue via a five-membered cyclic intermediate (*9*−*11*). With this presumptive mechanism and the optimum for enzyme activity near neutral pH, it is reasonable to postulate that 1 or more of the 10 QC histidine residues participates in catalysis. This paper describes the use of diethyl pyrocarbonate and site-directed mutagenesis to identify histidine residues essential to the enzymatic activity of human glutaminyl cyclase.

MATERIALS AND METHODS

Materials. Diethyl pyrocarbonate (DEPC), imidazole, hydroxylamine, glutamate dehydrogenase, ampicillin, and chloramphenicol were from Sigma. Glutamine-amide (Gln-NH_2) was from Bachem. If not otherwise stated, the chemicals were of analytical grade. Papaya glutaminyl cyclase was prepared as previously reported (*10*). Glutaminyl cyclase activity was determined using the coupled spectrophotometric assay previously described (*12*). Protein was quantitated with the Pierce Coomassie reagent using bovine serum albumin as a standard. Denaturing gel electrophoresis was performed using a mini-gel system and 12% Tris−glycine Novex precast gels, and proteins were visualized using Coomassie blue staining. The glutathione-*S*-transferase−human pituitary glutaminyl cyclase fusion protein was used in all studies below and was expressed using the Pharmacia pGEX-4T-2 system as previously reported (*9*). Briefly, the human QC cDNA (*8*) was fused to that of *S. japonicum* glutathione-*S*-transferase at the QC amino terminus, beginning at the codon corresponding to alanine 35 to remove the native signal sequence. *E. coli* BL21(DE3)pLysS cells were transformed with this construct, cells were grown to midlog phase, and enzyme was expressed upon induction with 0.1 mM IPTG for 3 h at 37 °C. Cells were harvested, lysed, and enzyme purified over GST-Sepharose using the manufacturer's standard protocol. Although efforts to remove the GST with thrombin have been unsuccessful, the expressed enzyme exhibited K_m values for glutaminamide (1.0 mM) and Gln-Gln (0.6 mM) that were identical to those for the native enzyme extracted from bovine pituitary (*12*). It is noted for the intrinsic fluorescence studies below that GST contains one tryptophan while QC contains seven and thus dominates the fluorescence observed.

DEPC Inactivation Protocol. Diethyl pyrocarbonate was prepared as a stock solution in absolute ethanol and quantitated by the absorbance increase at 240 nm upon incubation with imidazole (*13*). Reaction with QC was initiated with a 10-fold dilution of DEPC stock into a QC preparation. This was incubated for fixed time intervals at 30 °C, and the reaction was quenched by the removal of 50 μL aliquots of the incubation mixture into 20 μL of 20 mM imidazole at pH 7. Measurement of residual QC activity was

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[1] Abbreviations: QC, glutaminyl cyclase; pGlu, pyroglutamyl residue; DEPC, diethyl pyrocarbonate; GST, glutathione-*S*-transferase; MES, 2-morpholinoethanesulfonic acid; MOPS, 2-morpholinopropanesulfonic acid.

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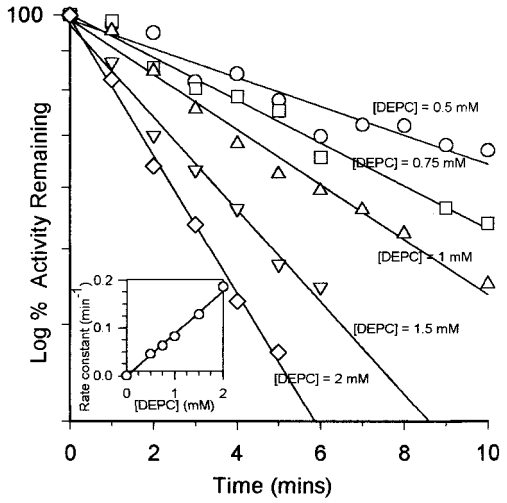

FIGURE 1: Time-dependent inactivation of QC. Glutaminyl cyclase was exposed to the indicated concentrations of DEPC and residual enzyme activity monitored as a function of exposure time. Inset: Replot of the first-order rate constants derived from the same data against DEPC concentration. Details are under Materials and Methods.

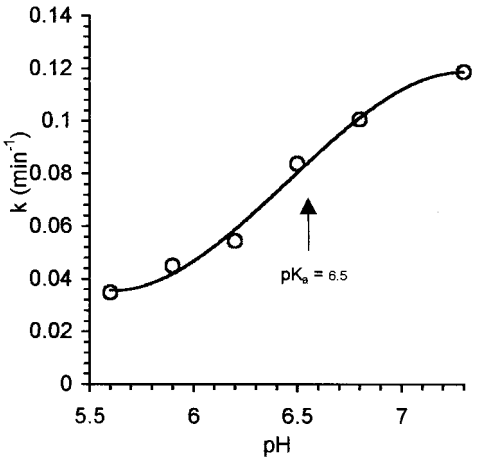

FIGURE 2: pH dependence of QC inactivation. Glutaminyl cyclase was inactivated by 1.5 mM DEPC at several pH values. The first-order rate constant of inactivation is shown as a function of pH. A standard titration curve was fit to the data with Sigmaplot.

accomplished by addition of 100 μL of assay cocktail to the quenched reaction followed by incubation and measurement using the standard protocol. The inactivation buffer was a combination of 0.1 M MES, 0.1 M MOPS, and 0.1 M boric acid (MMB buffer) to facilitate pH dependence studies.

Wavelength Scan and Stoichiometry of DEPC-Modified QC. To observe spectrophotometrically the results of DEPC inactivation, the protocol from Bateman and Hersh (*13*) was adopted. Briefly, 0.7 mL of QC (0.15 mg/mL), pH 7, was exposed to 1.5 mM DEPC and the solution scanned from 200 to 300 nm at 1 min intervals for 10 min.

The stoichiometry of histidine modification was measured in the same manner except the absorbance at 240 nm was followed continuously for 20 min. To correlate histidine modification with enzyme inactivation, an identical reaction was performed outside the spectrophotometer. Aliquots were removed every minute and quenched, and enzyme activity was measured as described in the DEPC inactivation protocol above.

Site-Directed Mutagenesis. Mutants were generated with the USE mutagenesis kit from Amersham-Pharmacia Biotech. The sequences of the mutagenic primers are as follows: H140Q (AATATTTGGAGTCATACTGGCAGGC), H307Q (AAATGGAATTTGGTCATCCTG), H319Q (CGGTATCAGCTGCAGAACTGG), H330Q (TGGTTTGCCAGACCTCAGGG). The underlined letters represent the substituted nucleotides. Each mutant was verified by DNA sequencing. Proper protein expression was confirmed by SDS−PAGE and Western blotting (*8*).

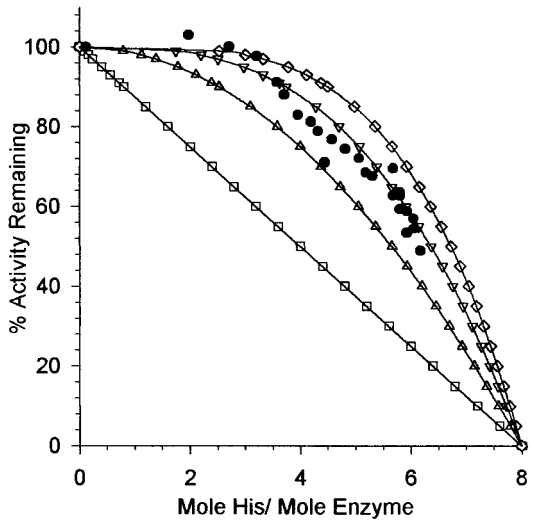

FIGURE 3: Stoichiometry of histidine modification. Inactivation of glutaminyl cyclase displayed as a function of histidine/enzyme molar stoichiometry. The theoretical number of essential histidines are represented by open squares (1), open triangles (2), open inverted triangles (3), and open diamonds (4). The closed circles are the experimental data points.

Fluorescence Studies of Mutants. Fluorescence studies of human QC were carried out essentially as described by Zerhouni et al. (*14*) for the papaya QC using *N*-acetyl-L-tryptophan ethyl ester (NATEE) as a fluorescence standard.

RESULTS

Inactivation of Glutaminyl Cyclase with Diethyl Pyrocarbonate. Recombinant human pituitary glutaminyl cyclase was rapidly inactivated by low concentrations of DEPC at neutral pH. The inactivation clearly proceeded by first-order kinetics. The first-order rate constant of inactivation was directly proportional to the DEPC concentration (Figure 1), yielding a second-order rate constant for inactivation at pH 7.8 and 30 °C of 91 M^{-1} min^{-1}. This inactivation rate is comparable to those reported for heparinase II (160 M^{-1} min^{-1}, ref *15*), 1-aminocyclopropane-1-carboxylic acid oxidase (170 M^{-1} min^{-1}, ref *16*), and dehydroquinase (148 M^{-1} min^{-1}, ref *17*). The pH dependence of inactivation could be fit to the titration of a reactive residue with an apparent pK_a of 6.5 (Figure 2). Inactivation of partially purified bovine QC (*12*) in parallel with the recombinant human QC showed identical inactivation kinetics (data not shown).

Monitoring of the UV absorbance spectrum under the reaction conditions resulted in an increase in absorbance at 240 nm typical of histidine modification, with no absorbance change at 280 nm that would indicate tyrosine modification. Quantitative analysis of the histidines modified correlated with inactivation rate by the method of Tsou (*18*) yielded a total of 6−8 histidines modified, with 3 of these being apparently essential for activity (Figure 3). The number of

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H. sapiens          (1)  MAGGRHRRVV GTLHLLLLVA ALPWASRGVS PS----ASAW PEEKNYHQPA
D. melanogaster     (1)  -MLHRTARMW TLCVQTALIA TLVRGSTSQK DN------LV GRTQISYNPS
B. jararaca         (1)  MARERRDSKA ATFFCLAWAL CLALPGYPQH VSGREDRADW TQEKYSHRPT
C. elegans          (1)  ---------- ---MGLALVL GILICTTSAW GQ-------W RTNQRTHQLS
S. cerevisiae       (1)  ----MGMKYV LPLRLIGLAY LLVLFQVHRV TG-------W ELSYEQYHAA

H. sapiens         (47)  ILNSSALRQI AEGTSISEMW QNDLQPLLIE RYPGSPGSYA ARQHIMQRIQ
D. melanogaster    (44)  ELSEPRFLEY SN-LSDKLHL REAIDKILIP RVVGTTNHSI VREYIVQSLR
B. jararaca        (51)  ILNATCILQV TSQTNVSRMW QNDLHPILIE RYPGSPGSYA VRQHIKHRLQ
C. elegans         (31)  LLPESSTLRL CRDFTNTTRF KEILAPIMVP RIVDTKQHRQ VGDYLQSFLH
S. cerevisiae      (41)  HLN----EAI NPDSGWNKST KNLLLPFNRT RVPGSEGSRE IQRFIIEHFN

H. sapiens         (97)  RLQADWVLEI DTFLSQTPYG -YRSFSNIIS TLNPTAKRHL VLACHYDSKY
D. melanogaster    (93)  --DLDWDVEV NSFHDHAPIK GKLHFHNIIA TLNPNAERYL VLSCHYDSKY
B. jararaca       (101)  GLQAGWLVEE DTFQSHTPYG -YRTFSNIIS TLNPLAKRHL VIACHYDSKY
C. elegans         (81)  N--LGFATEW DAFTDTTPLG -TRNFRNLIA TFDESAPRRL VLACHYDSKI
S. cerevisiae      (86)  NTLAGEWAVE TQAFEENGYR ----FNNLVM TLQNNASEYL VLAAHYDTKI

H. sapiens        (146)  FS-HWNNRVF VGATDSAVPC AMMLELARAL DKKLLSLK-T VS--------
D. melanogaster   (141)  MP----GVEF LGATDSAVPC AMLLNLAQVL QEQLKPLK-- ----------
B. jararaca       (150)  FPPQLDGKVF VGATDSAVPC AMMLELARSL DRPLSFLKQS SL--------
C. elegans        (128)  IP----GQVM IAATDSAVPC AMMLDIAQTL APYMYKRV-- -A--------
S. cerevisiae     (132)  AP-----TGM VGAIDSAASC AALLYTAQFL THIACHERTK EYNDLESNTV

H. sapiens        (186)  DSKPDLSLQL IFFDGEEAFL HWSPQDSLYG SRHLAAKMAS TPHPPGA---
D. melanogaster   (175)  --KSKLSLML LFFDGEEAFE EWGPKDSIYG ARHLAKKWH- --HEG-----
B. jararaca       (192)  PPKADLSLKL IFFDGEEAFV RWSPSDSLYG SRSLAQKMAS TPHPPGA---
C. elegans        (163)  ---QQIGLQL IFFDGEEAFR DWTATDSLYG SRHLAQKWEQ KWYPSSSSLN
S. cerevisiae     (177)  VSNSTLGVKI VFFDGEEAIE EWGPEDSIYG ARRLAAQWLA D---------

H. sapiens        (233)  --RGTSQLHG MDLLVLLDLI GAPN--PTFP NFFPN-SARW FERLQAIEHE
D. melanogaster   (215)  )------KLDR IDMLVLLDLL GAPD--PAFY SFFEN-TESW YMRIQSVETR
B. jararaca       (239)  --RNTYQIRG IDLFVLLDLI GARN--PVFP VYFLN-TARW FGRLEAIERN
C. elegans        (210)  NFELSKELDR IDVLMLLDLL GAAN--PSIG NTIGMGANDL FSQLADVESN
S. cerevisiae     (218)  G-----TMTR IRLLFLLDLL GSGEEEPLVP SYYAE-THQE YQLLNRIEDD

H. sapiens        (278)  LHELGLLKDH SLE------- GRY------- FQNYSYG-GV IQDDHIPFLR
D. melanogaster   (256)  LAKLQLLERY ASSGVAQRDP TRY------- FQSQAMRSSF IEDDHIPFLR
B. jararaca       (284)  LNDLGLLNNY SSE------- RQY------- FRSNLRR-HP VEDDHIPFLR
C. elegans        (258)  LRTSGCLSSL RRN------- ---------- VFNKQLSYNQ VEDDHIPFLK
S. cerevisiae     (262)  LLFRRGDEIN GESALAAEVA RQRKHLDPTD YRFLGLGHSV IGDDHTPFLA

H. sapiens        (313)  RGVPVLHLIP SPFPEVWHTM DDNEENLDES TIDNLNKILQ VFVLEYLHL-
D. melanogaster   (299)  RNVPILHLIP VPFPSVWHTP DDNASVIDYA TTDNLALIIR LFALEYLLAG
B. jararaca       (319)  RGVPILHLIP SPFPRVWHTM EDNEENLDKP TIDNLSKILQ VFVLEYLNLG
C. elegans        (291)  RGVPILHLIT VPFPSVWHRS SDNANALHYP TIDHMTAVIR VFVAKYLGIA
S. cerevisiae     (312)  AGVPVLHAIP LPFPSTWHTV DDDFRHLDAA ETRHWALLVC EFVVQSLRSR

H. sapiens        (362)  ----
D. melanogaster   (349)  TEAK
B. jararaca       (369)  ----
C. elegans        (343)  PA--
S. cerevisiae     (364)  NQ--
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FIGURE 4: QC protein sequence alignment. Multiple alignment of human glutaminyl cyclase protein sequence (GenBank CAA50438.1) with apparent homologues from fruitfly (AAF51611.1), snake (BAA34290), worm (T23125), and yeast (P43599). The shaded areas represent identical residues.

essential residues remained constant even when the inactivation pH was varied from 6.5 to 7.8. The inactivated enzyme could be partially reactivated with hydroxylamine. Enzyme inactivated to 17% residual activity could be reactivated to 44% residual activity with 2 mM hydroxylamine. Higher concentrations of hydroxylamine could not be used because of interference with the enzyme assay. It is also noted that substrates could not be used to block the active site from inactivation since all substrates contained a primary amine of relatively low pK_a, i.e., the amino terminus, and would thus react with the reagent itself.

Mutation of Conserved Histidines. Figure 4 shows an alignment of the protein sequence of QC across several eukaryotic species from humans to yeast, with blocks indicating the 63 identical residues including 4 histidines (140, 307, 319, 330). Mutation of these histidine residues to glutamine yielded two mutant enzymes with little or no measurable enzymatic activity (H140Q and H330Q) and two enzymes with increased K_m values but little change in k_{cat} (H307Q and H319Q) (Table 1). Fluorescence emission spectra (excitation 295 nm) of both mutated and unmutated QC forms were identical, with a peak at 338 nm. Acrylamide quenching of endogenous tryptophan fluorescence was also identical between the mutant and unmutated enzymes (Figure 5), indicating the tryptophan environments were not altered and therefore no significant structural changes had occurred upon mutagenesis.

Table 1: Analysis of Individual Histidine to Glutamine Mutations in QC[a]

	kinetic analysis of histidine mutants	
	k_{cat} (min^{-1})	K_m (mM)
unmutated	26.1 ± 6.4	1.1 ± 0.1
H307Q	23.3 ± 0.8	4.3 ± 0.6
H319Q	21.6 ± 2.6	4.9 ± 1.6

[a] Analysis was performed at pH 7.8 and 37 °C using the substrate glutaminamide. Numbers represent mean ± SEM of at least three independent experiments. Mutants H140Q and H330Q were not active enough to obtain reliable kinetic constants.

DISCUSSION

Enzymatic cyclization of glutamine to pyroglutamic acid is a simple transamidation reaction that can be envisioned

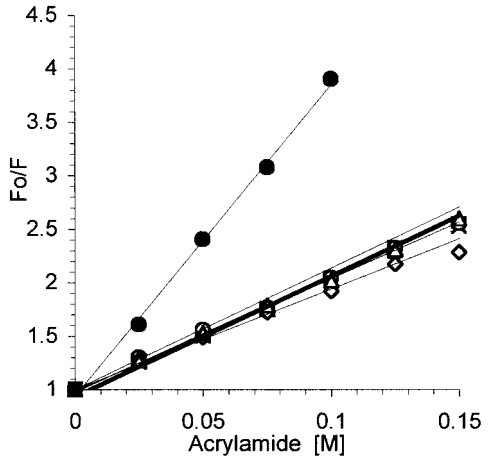

FIGURE 5: Acrylamide quenching of tryptophan fluorescence. The ratio of initial fluorescence to fluorescence in the presence of acrylamide is shown as a function of acrylamide concentration. Fluorescence intensities were determined at 356 nm for the standard, *N*-acetyltryptophan ethyl ester (closed circles), and at 338 nm for QC in 50 mM sodium phosphate buffer (pH 7 and 25 °C) after excitation at 295 nm. The QC mutants are shown as open squares (His 330), open circles (His 317), open diamonds (His 319), open triangles (His 140), and the cross (unmutated).

to proceed via either a nucleophilic or an acid/base mechanism. Previous studies have shown that mammalian glutaminyl cyclase does not possess the reactive serine or cysteine residues (*4*, *9*) that one would expect for nucleophilic catalysis. Likewise, plant (papaya) QC lacks an apparent nucleophile, and inhibition by proline-containing dipeptides indicates a direct cyclization mechanism (*10*, *11*) for this enzyme.

One obvious candidate for an active site residue which participates in promotion of direct glutamine cyclization by an acid/base catalysis is histidine. Such participation by histidine was confirmed in this study by inactivation of human QC upon exposure to diethyl pyrocarbonate. The rate of inactivation at neutral pH, the characteristic absorption peak produced at 240 nm, the apparent pK_a of modification (6.5), and partial reactivation with low concentrations of hydroxylamine are all consistent with modification of essential histidine residues in human QC.

A comparison of the protein sequence of human QC with the putative homologues from snake, fruitfly, worm, and yeast shows 63 conserved residues comprising nearly 20% of the mature protein. Of the 16 histidines in the mature human QC, only 4 are completely conserved between species. An analysis of the stoichiometry of QC modification with DEPC in comparison to the reaction rate indicated a reaction of up to eight histidine residues, three of which appeared to be essential for enzyme activity. This could be interpreted as indicating only three of the conserved four histidines are essential, only three of the four are accessible to DEPC, or that only two histidines are essential and that modification of each of the other two yields partially active enzyme.

We favor this last interpretation for two reasons. First, our kinetic analysis of the four histidine mutants is consistent with two histidines (140 and 330) acting directly in catalysis and two others (307 and 319) participating in substrate binding. Modification of histidines 140 and 330, therefore, would be expected to result in an inactive enzyme. Modification of histidines 307 and 319 would influence activity by altering but not necessarily abolishing substrate binding. Second, QC is predicted by PRODOM (Release 2000.1) to contain an aminopeptidase domain. This is not altogether surprising since both QC and aminopeptidases recognize the amino termini of peptides and proteins. Although QC does not appear to contain aminopeptidase activity (unpublished results), QC and aminopeptidases could share some active site residues such as the histidines reported here. A prediction of the structural fold using 3D-PSSM, FUGUE, and 123D indicated that QC had a phosphorylase/hydrolase-like fold and was in the bacterial zinc aminopeptidase family. The prototype of this family is the *Aeromonas proteolytica* aminopeptidase, which contains two active site zinc atoms and folds into a single α/β globular domain with a central mixed sheet. Homology modeling of QC on the structure of *Aeromonas proteolytica* aminopeptidase (S. Lovell and R. Bateman, manuscript in preparation) revealed that the two histidine residues (H97 and H256) coordinating the active site zinc atoms in the aminopeptidase corresponded to histidines 140 and 330 in human QC, the same two proposed above to be direct participants in catalysis of glutamine cyclization. At present, QC does not appear to be a metalloenzyme based on the ability to completely recover enzyme activity after urea denaturation/renaturation (*4*), and lack of inhibition upon exposure to EDTA (*4*) or dialysis against *o*-phenanthroline (unpublished results). It is probable, then, that histidines 140 and 330 act as acid/base catalysts rather than zinc ligands in the glutamine cyclization reaction. QC histidines 307 and 319 correspond to H340 and M242 in the aminopepidase, both of which are present in the active site poised for substrate binding as shown by enzyme−inhibitor complexes (*19*, *20*). Histidine has also been shown to participate in substrate binding in aminopeptidases A, N, and P, as well as methionine aminopeptidase (*21*−*24*).

Previous studies have shown a number of similarities between mammalian QC and plant (papaya) QC including size, predicted glycosylation pattern, substrate specificity, pH optimum for enzyme activity, and intracellular location (*10*, *11*, *25*−*28*). However, there is a complete lack of sequence homology between the plant and animal glutaminyl cyclase families (*6*, *8*, *25*) even though the QC homologues are very closely related by sequence within families as seen in Table 1 and refs *6* and *9*. This would seem to indicate independent evolutionary origins for the two families. Further, the papaya QC is completely impervious to diethyl pyrocarbonate even at 5 mM (S. Misquitta and R. Bateman, unpublished results), a finding in stark contrast to the rapid inactivation of human QC with DEPC reported here. Finally, spectroscopic studies have suggested that papaya QC is an all-β structure (*29*) while secondary structure and fold prediction programs point to an α/β structure for mammalian QC. It appears likely, therefore, that the mammalian and plant glutaminyl cyclase families possess very different enzyme structures and utilize different catalytic strategies to produce amino-terminal pyroglutamyl residues in peptides and proteins.

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