

Identification of Human Glutaminyl Cyclase as a Metalloenzyme

POTENT INHIBITION BY IMIDAZOLE DERIVATIVES AND HETEROCYCLIC CHELATORS*

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Stephan Schilling‡, André J. Niestroj‡, Jens-Ulrich Rahfeld‡, Torsten Hoffmann‡,
Michael Wermann‡, Katrin Zunkel‡, Claus Wasternack§, and Hans-Ulrich Demuth‡¶

From the ‡Probiobio Aktiengesellschaft, Weinbergweg 22, and the §Leibniz Institute for Plant Biochemistry,
Post Office Box 110432, 06120 Halle/Saale, Germany

Human glutaminyl cyclase (QC) was identified as a metalloenzyme as suggested by the time-dependent inhibition by the heterocyclic chelators 1,10-phenanthroline and dipicolinic acid. The effect of EDTA on QC catalysis was negligible. Inactivated enzyme could be fully restored by the addition of Zn^{2+} in the presence of equimolar concentrations of EDTA. Little reactivation was observed with Co^{2+} and Mn^{2+} . Other metal ions such as K^+ , Ca^{2+} , and Ni^{2+} 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-[3-(1*H*-imidazol-1-yl)propyl]-2-thioxoimidazolidin-4-one and 1,4-bis-(imidazol-1-yl)-methyl-2,5-dimethylbenzene, which exhibited respective K_i 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 kinetically obtained pK_a 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 pK_a determination, indicating the requirement for an unprotonated nitrogen for binding to QC. Similarly, the pH dependence of the kinetic parameter K_m for the QC-catalyzed conversion of H-Gln-7-amino-4-methylcoumarin also implies that only N-terminally 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-dependent transferase, suggesting that the active site-bound metal is a potential site for interaction with novel, highly potent competitive inhibitors.

Glutaminyl cyclases (QC)¹ (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 pyrogluta-

mate from glutamine was assumed to proceed spontaneously (1). However, the QCs were identified more recently as catalysts of the reaction in both mammals and plants (2–5). Generally, QCs from both mammalian and plant sources appear to be very similar monomeric proteins that are expressed in the secretory pathways and have similar molecular masses, ~33 and ~40 kDa, respectively (6, 7). Their primary structures, however, reveal no sequence homology, and the enzymes feature completely different folding patterns. Whereas plant QC consists almost solely of β -sheet structure, mammalian QCs are predicted to possess an α/β -fold (8–10). Furthermore, plant QC does not share sequence or structural homology to other plant enzymes, belonging, apparently, to a separate enzyme subfamily (4). Mammalian QCs, however, exhibit remarkable homology toward bacterial aminopeptidases, suggesting their evolutionary origin in this protein family (9).

Investigating the substrate specificity of both enzymes, we recently found differences between papaya and human QC (24). Although the catalysis of cyclization of and the inhibition by modified N-terminal residues were quite different, both enzymes catalyzed the cyclization of oligopeptides with similar specificity rate constants. Moreover, they also catalyzed the formation of a five-membered lactam ring from L- β -homoglutaminyl peptides. Based on the present state of knowledge, it is assumed that plant and animal QCs catalyze the formation of N-terminal pyroglutamic acid (pGlu) residues by enabling the intramolecular cyclization of the glutaminyl residue in a non-covalent manner (11, 12). However, the results of the substrate specificity study revealed differences in substrate recognition by both enzymes.

Thus, a more detailed analysis of the inhibition pattern of plant and human QCs should help to deepen our understanding of QC catalysis. To date, however, systematic inhibitor data exist only for plant QC, which is competitively inhibited by peptides containing an N-terminal proline residue (13), whereas human QC was shown to be inactivated by 1,10-phenanthroline and reduced 6-methylpterin (3). 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.

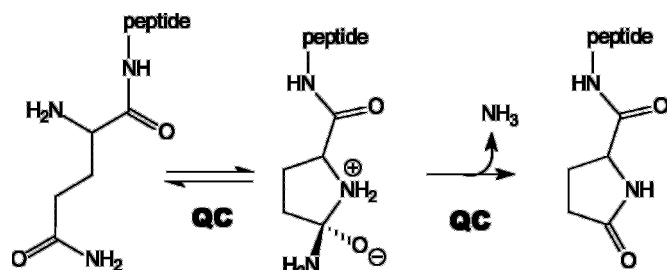
EXPERIMENTAL PROCEDURES

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, pyroglutamyl aminopeptidase came from Qiagen, H-Gln-AMC and H-Gln-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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¶ To whom correspondence should be addressed. Tel.: 49-345-5559900; Fax: 49-345-5559901; E-mail: hans-ulrich.demuth@probiobio.de.

¹ The abbreviations used are: QC, glutaminyl cyclase; AMC, 7-amino-4-methylcoumarin; Mes, 4-morpholineethanesulfonic acid.



SCHEME 1. N-terminal cyclization of glutamyl peptides by QC.

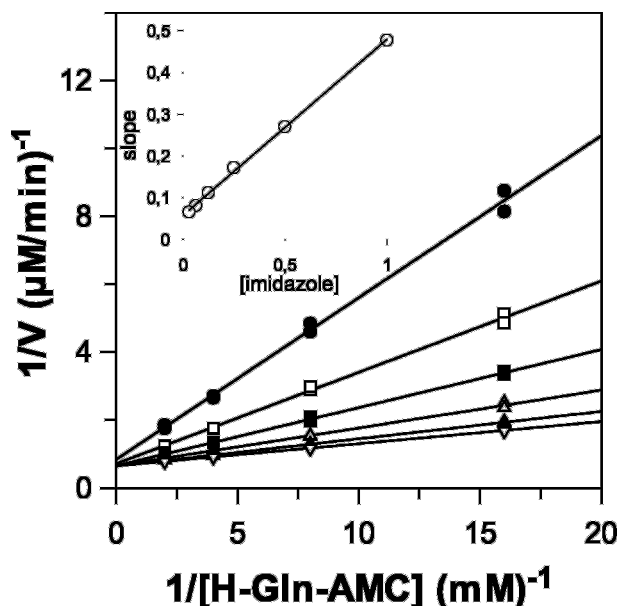


FIG. 1. Lineweaver-Burk plots for human QC catalyzed cyclization of H-Gln-AMC in presence of various concentrations of imidazole between 0.03 and 1 mM. The inset shows a secondary plot of the obtained slopes of the Lineweaver-Burk evaluation versus the inhibitor concentrations. The conditions were 0.05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

Aldrich, Asinex, TimTec, and InterBioScreen. Papaya QC was purified from crude papain as described previously (14). All other chemicals were of analytical or high pressure liquid chromatography grade.

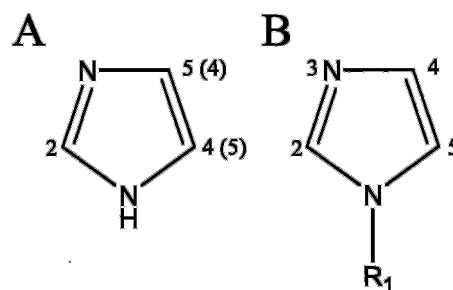
QC Assays—QC activity was assayed essentially as described elsewhere (14). Briefly, the assay reactions (250 μ l) consisted of varying concentrations (~ 0.25 – $4 K_m$) of H-Gln-AMC or H-Gln-Gln-OH in 0.05 M Tris-HCl, pH 8.0. In some cases, assay buffer also contained up to 5 mM EDTA, which has been shown to stabilize the auxiliary enzyme (15). K_i values of QC inhibition were not influenced by EDTA. In the case of the spectrophotometric assay, samples additionally contained 30 units/ml glutamic acid dehydrogenase, 0.25 mM NADH/ H^+ , and 15 mM α -ketoglutaric acid. Reactions were started by the addition of QC, and activity was monitored by recording the decrease in absorbance at 340 nm. In contrast, the assay samples for the fluorometric detection of QC activity contained only buffer and 0.4 units/ml pyroglutamyl aminopeptidase as an auxiliary enzyme. The excitation/emission wavelengths were 380/460 nm. Reactions were started by the addition of QC. QC activity was determined from a standard curve of AMC under assay conditions. All determinations were carried out at 30 °C using either the TECAN SpectraFluor Plus or the BMG Novostar reader for microplates.

Inhibitor 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

TABLE I
Inhibition constants of imidazole derivatives in the human QC catalyzed reaction

Assays were performed at 30 °C in 0.05 M Tris-HCl, pH 8.

Compound	K_i value μ M
Core structures	
Imidazole	103 \pm 4
Benzimidazole	138 \pm 5
N-1 derivatives	
1-Benzylimidazole	7.1 \pm 0.3
1-Methylimidazole	30 \pm 1
1-Vinylimidazole	49 \pm 2
Oxalic acid diimidazolidide	78 \pm 2
N-Acetylimidazole	107 \pm 3
N-(Trimethylsilyl)-imidazole	167 \pm 7
N-Benzoylimidazole	174 \pm 7
1-(2-Oxo-2-phenylethyl)-imidazole	184 \pm 5
1-(3-Aminopropyl)-imidazole	410 \pm 10
1-Phenylimidazole	No inhibition
1,1'-Sulfonyldiimidazole	No inhibition
C-4 (5) derivatives	
N- ω -acetylhistamine	17 \pm 1
L-Histidinamide	560 \pm 40
H-His-Trp-OH	600 \pm 30
L-Histidinol	1530 \pm 120
L-Histidine	4400 \pm 200
4-Imidazole-carboxaldehyde	7600 \pm 700
Imidazol-4-carbonic acid methylester	14500 \pm 600
C-4,5 derivatives	
5-Hydroxymethyl-4-methyl-imidazole	129 \pm 5
5-Amino-3H-imidazole-4-carboxylic acid amide	15500 \pm 500
4,5-Diphenyl-imidazole	No inhibition
4,5-Dicyanoimidazole	No inhibition
C-2 derivatives	
2-Methyl-benzylimidazole	165 \pm 4
2-Ethyl-4-methyl-imidazole	580 \pm 40
2-Aminobenzimidazole	1800 \pm 100
2-Chloro-1H-benzimidazole	No inhibition



SCHEME 2. The constitution of the imidazole ring (A) and an imidazole N-1 derivative (B).

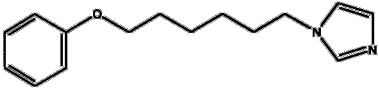
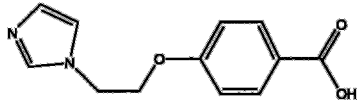
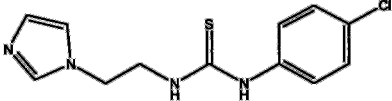
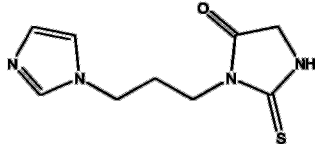
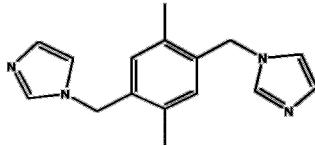
GraFit software (version 5.0.4. for windows, Erithacus Software Ltd., Horley, UK).

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 0.06 M acetic acid, 0.06 M Mes, and 0.12 M Tris (16). 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 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 parameter K_m using GraFit software. Because of the reduced stability and catalytic activity of the auxiliary enzyme pyroglutamyl aminopeptidase under acidic and basic conditions, the study was limited to the range between 5.5 and 8.5 pH.

Enzyme Inactivation/Reactivation Procedures—An aliquot of human QC (0.1–0.5 mg, 1 mg/ml) was inactivated overnight by dialysis against a 3000-fold excess of 5 mM 1,10-phenanthroline, or 5 mM dipicolinic acid in 0.05 M Bis-Tris/HCl, pH 6.8. Subsequently, the inactivating agent was carefully removed by dialysis (three cycles, 2000-fold excess) of the samples against 0.05 M Bis-Tris/HCl, pH 6.8, containing 1 mM EDTA.

TABLE II
Inhibition of human QC by N1 derivatives selected from compound databases

Determinations were carried out as described in Table I.

Compound	K_i -value	Structure
1-(6-phenoxyhexyl)-1 <i>H</i> -imidazole	$6.2 \pm 0.03 \mu\text{M}$	
4-(2-imidazol-1-yl-ethoxy)-benzoic acid	$2.3 \pm 0.03 \mu\text{M}$	
<i>N</i> -(4-chlorophenyl)- <i>N</i> ^ε -[2-(1 <i>H</i> -imidazol-1-yl)ethyl]thiourea	$1.2 \pm 0.03 \mu\text{M}$	
3-[3-(1 <i>H</i> -imidazol-1-yl)propyl]-2-thioxoimidazolidin-4-one	$818 \pm 1 \text{ nM}$	
1,4-bis-(imidazol-1-yl)methyl-2,5-dimethylbenzene	$295 \pm 5 \text{ nM}$	

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 103 ± 4 and $129 \pm 8 \mu\text{M}$ 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 $138 \pm 5 \mu\text{M}$. Obviously, the condensed ring structure does not influence significantly the binding of the compound under the conditions used. Driven by the similar inhibitory characteristics of imidazole and benzimidazole, derivatives of both compounds were tested to identify secondary interactions that improve or diminish their inhibitory potency.

Imidazole Derivatives—Imidazole and benzimidazole derivatives carrying substituents at different positions of the ring system were tested, and the influence of the substituents was

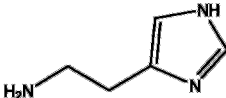
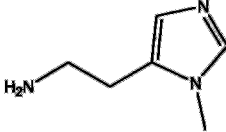
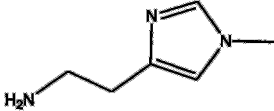
compared (Table I). The constitution refers to the imidazole ring (Scheme 2).

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-methyl-imidazole 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

TABLE III
Inhibition of human QC by L-histamine and two derivatives

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.

Compound	K_i -value (μ M)	Structure
L-histamine	850 ± 40	
1-methyl-5-(β -aminoethyl)-imidazole	120 ± 4	
1-methyl-4-(β -aminoethyl)-imidazole	n.i.	

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.

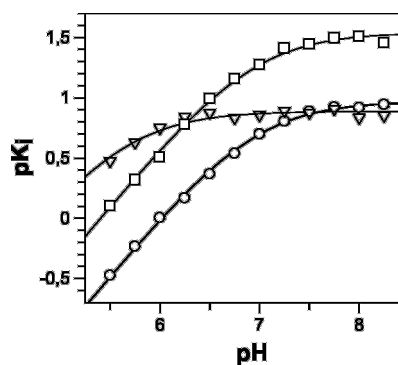


FIG. 2. The pH-dependence of the inhibition constant of imidazole (circles), benzimidazole (triangles), and 1-methylimidazole (squares) inhibiting human QC catalyzed cyclization of H-Gln-AMC. Data points were fitted to a single dissociation model and revealed pK_a values of 6.94 ± 0.02 , 6.93 ± 0.03 and 5.60 ± 0.05 for imidazole, 1-methylimidazole, and benzimidazole, respectively. Reactions were carried out at 30 °C in 0.06 M acetic acid, 0.06 M Mes, and 0.12 M Tris adjusted to the respective pH by the addition of NaOH or HCl.

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 IV
Comparison of the dissociation constants of inhibitors and a substrate determined by the pH-dependence of inhibitory and Michaelis-Menten constants as well as by acid/base titration

Assays were carried out at 30 °C in 0.06 M acetic acid, 0.06 M Mes, and 0.12 M Tris. The turnover number was found to be constant between pH 5.5 and 8.5. $K_{M(I)}$ reflects the dissociation of the substrate. $K_{M(II)}$ reveals a group of the enzyme.

Compound	Parameter	pK_a kinetic determination	pK_a titrimetric determination
Imidazole	K_i	6.94 ± 0.02	6.946 ± 0.003
Benzimidazole	K_i	5.60 ± 0.05	5.500 ± 0.010
1-Methylimidazole	K_i	6.93 ± 0.03	7.000 ± 0.003
H-Gln-AMC	$K_{M(I)}$	6.81 ± 0.04	6.830 ± 0.010
	$K_{M(II)}$	8.60 ± 0.10	—

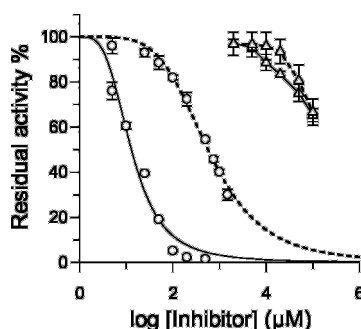


FIG. 3. Inhibition of human QC by metal-chelating reagents. Concentration dependence of inhibition by 1,10-phenanthroline (circles) and EDTA (triangles) is shown. Residual activity of QC in the presence of either compound was determined directly after the addition (dotted traces) or preincubation of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

inhibition 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 H-Gln-AMC (Table IV). In the basic pH range, data revealed a pK_a value of 8.6 ± 0.1 , apparently reflecting a dissociating group of the QC protein.

Inhibition by Heterocyclic Chelators—The inhibition of porcine QC by 1,10-phenanthroline has already been described (3). However, the fact that EDTA has been shown to have an activating effect on QC catalysis suggested that inhibition by phenanthroline is not due to metal chelation (3). Also, in addition to being inhibited by 1,10-phenanthroline, human QC-catalyzed substrate cyclization was abolished in presence of dipicolinic acid, another inhibitor of metalloenzymes. Both chelators inhibited QC in a competitive and time-dependent manner, *i.e.* initial activity that was already competitively inhibited was found to be further reduced after prolonged incubation with the compounds (Fig. 3). Interestingly, EDTA did not show remarkable inhibition regardless of incubation time or under any conditions.

Human QC was almost completely inactivated after extensive dialysis against 5 mM 1,10-phenanthroline or 5 mM dipicolinic acid. After repeated dialysis overnight against chelator-free buffer solutions, QC activity was partially reactivated up to 50–60% (data not shown). However, when dialyzed against buffers containing 1 mM EDTA, no reactivation was observed.

Near-total restoration of QC activity after inactivation by

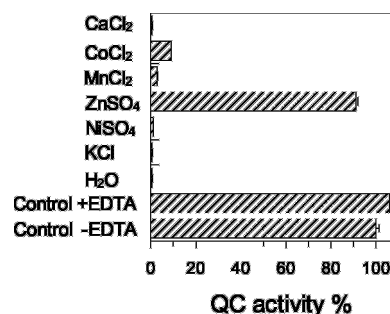


FIG. 4. Reactivation of human QC with monovalent and divalent metal ions. QC was nearly inactivated by the addition of 2 mM dipicolinic acid in 50 mM Bis-Tris, pH 6.8. Subsequently, the enzyme was subjected to dialysis against 50 mM Bis-Tris, pH 6.8, containing 1 mM EDTA. Reactivation of the enzyme was 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 unspecific 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).

either dipicolinic acid or 1,10-phenanthroline was achieved by incubating the protein for 10 min with 0.5 mM $ZnSO_4$ in presence of 0.5 mM EDTA (Fig. 4). Partial restoration of QC activity was similarly obtained using Co^{2+} and Mn^{2+} ions for reactivation. Even in the presence of 0.25 mM Zn^{2+} , a reactivation to 25% of the original activity was possible (data not shown). No reactivation was observed applying Ni^{2+} , Ca^{2+} , or K^+ ions. Similarly, incubation of fully active QC with these ions had no effect on the enzyme activity.

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

hQC	MAGGRHRRVVGTLLHLLLVAALPWASRGVSP SASANPEEKNYHQPAILNSSLARQIAEGT
SGAP	-----APDIPLANVKAHLTQLS
hGCP II	-----ANEYAYRRGIAEAVGLPSIPVHPIGYDDAQ-K
	* * : .
hQC	SISEM Q NDLQPLLIERYPGSPGSYAARQHIMQRIQRLQADWVLEIDTFLSQTPTYGYRSF
SGAP	TIAAN--NGGN----RAHGRPGYKASVDYVKAKLD--AAGYTTTLQQFTSGGATGYNLI
hGCP II	LLEKM--GGSAP---PDSSWRGSLKVPYNVGPFGFTGNFSTQKVKMHIHSTNEVTIRYINVI
	: : *
hQC	SNIISTLNPTAKRHLVLACHYDSKYFSHNNRVFVGATD S AVPCAMMLELAR---ALDKK
SGAP	ANWPG-GDP--NKVLMAGAH L DS--VSSG----AGINDNGSGSAAVLETAL---AVSRA
hGCP II	GTLRGAVEP--DRYVILGGH R DS-----W---VFGGIDPQSGAAVVHEIVRSFGTLKKE
	. . . : * . : : . * ** * * . * : * . : : .
hQC	LLSLKTVSDSKPDLSLQLIFFDGE E AFLHWS PQDSLYGSRHLAAKMASTPHPPGARGTSQ
SGAP	GY--Q-----PDKHLRFAWUGAE E LGLIGS---KFY-----VMNLPSADR--SK
hGCP II	GW--R-----PRRTILFASUDAE E FGLLGS---TEW-----AEENSR-LLQ
	: * : : . . * * * . : * :
hQC	LHGMDLLVLD L LIGAPNPTFPNFF--PNSARUFERLQAIEHELH---ELGLLKDHSLEGR
SGAP	LAG---YLNFD M IGSPNPGYFVYDDDPVIEKTFKNYFAGLNVP T ---EIE T EGDGRSDHA
hGCP II	ERG-VAYINAD S IEGNYTLRV D CT-PLMYSLVHNLTKELKSPDEGFEGKSLYESWT K KS
	* : * * * . . : * : .
hQC	Y---FQNYSY---G-G-----VIQDD-HIPFLRRGV P -VLHLIPSPFPEV W HTMDDNEE
SGAP	P---FKNVGV P --VG-G-----LFTGAGYTKSAAQAQK-WGGTAGQAFDRCY H SSCDSLS
hGCP II	PSPEFGM P RISKLGSGND FEVFFQRLGIASGRARYTKN W ETNKFSGY P -LY H SVYETYE
	* . . * * . : : . : * : . .
hQC	NLDESTID-N-LNKILQVFVLEYLHL----
SGAP	NINDTALDRNSDAAAHAINTLSSGTCEPPT
hGCP II	-LVEKFYD--PMFKYHLTV A QVRGGMV F EL
	: : . *

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).

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 metallo 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 metallo peptidases, 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-

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 γ -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 γ -amide group of the substrate glutaminyl residue, simultaneously stabilizing the oxanion formed by the nucleophilic attack of the α -nitrogen on the scissile γ -carbonyl carbon. Such an interaction of the γ -carbonyl group of the substrate and the active-site metal ion is also corroborated by the observed inhibition of human QC by γ -hydrazide residues. Furthermore, the interaction of one active site zinc ion with the α -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 mM 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 an acyltransferase, the already weak binding second Zn^{2+} ion was lost (20) because a further Zn^{2+} ion was not needed for exerting glutaminyl cyclization. The occupation of such a binding site at 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, 23), 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 α -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 reac-

tion 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 *N*-1 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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