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-NH_2, 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 pyroglutamate 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 abundancy 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 intracellulary 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

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[1] Abbreviations: BMGY, buffered glycerol complex medium; BMMY, buffered methanol complex medium; DAHC, diammonium hydrogen citrate; DAP A, dipeptidyl aminopeptidase A; DHAP, 2′,6′-dihydroxyacetophenone; DTT, dithiothreitol; GdmCl, guanidinium chloride; GlcNAc, *N*-acetyl-D-glucosamine; H-Gln-AMC, L-glutaminyl-4-methylcoumarinylamide; H-Gln-βNA, L-glutaminyl-2-naphthylamide; IMAC, immobilized metal ion affinity chromatography; IPTG, isopropyl β-D-1-thiogalactopyranoside; Man, D-mannose; MCP, monocyte chemotactic protein; QC, glutaminyl cyclase; TFA, trifluoroacetic acid; TRH, thyrotropin releasing hormone

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

oligonucleotiode	sequence (5′ → 3′), restriction sites (underlined)	restriction enzyme for cloning
QC−SDMCs	GCCGTGCCATGTGCAATGATGTTG	−
QC−SDMCas	CAACATCATTGCACATGGCACGGC	−
QC4	ATAGTCGACGCAGGCGGAAGACACCGGC	*Sal*I
QC5	ATAAAGCTTTTACAAATGAAGATATTCC	*Hin*dIII
pQCyc-1	ATATAGCATGCGGAGGAGAAGAATTACCACCAG	*Sph*I
pQCyc-2	ATATAAAGCTTACAAATGAAGATATTCCAACAC	*Hin*dIII
QC−Pic1	ATGCTAGCGCCTGGCCAGAGGAGAAGAAT	*Nhe*I
QC−Pic2	ATTCTAGAGTATTACAAATGAAGATATTC	*Xba*I
HPic-K1	GCTCATCATCATCATCATCATGCTAGCGGTAC	*Nhe*I
HPic-K2	CGCTAGCATGATGATGATGATGATGAGCTGCA	*Nhe*I

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 intracellulary 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 amplifications were performed according to the instructions of the supplier of the *Pfu* polymerase utilized (New England Biolabs). In brief, the complementary oligonucleotides QC−SDMCs and QC−SDMCas (Table 1) were employed for amplification of two fragments of the cDNA with oligonucleotides QC4 and QC5, which cover the open reading frame of QC. Subsequently, a second PCR was performed using the two fragments as template and again Primers QC4 and QC5, yielding the cDNA encoding a Cys in codon 164. The open reading frame was subcloned into the pPCR−Script Cam SK(+) vector applying the manufacturer's recommendations (Stratagene).

Molecular cloning of plasmid vectors encoding the human QC. All cloning procedures were done applying standard molecular biology techniques (*20*). For expression in yeast, the vector pPICZαB (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 *Pst*I and *Kpn*I. In addition, with the cassette a novel restriction site for *Nhe*I 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 pPCRScript Cam SK (+) vector and insertion into the yeast expression plasmid via the *Nhe*I and *Xba*I 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 *Sph*I and *Hin*dIII. 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, pPICZαB, three restriction sites are provided for linearization. Since *Sac*I and *Bst*XI cut within the QC cDNA, *Pme*I was chosen for linearization. 20−30 μg plasmid DNA was linearized with *Pme*I, 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

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 Ni^{2+}-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 OD_{600} 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 Ni^{2+}-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-NH_2. 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-NH_2. 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% tris-isopropylsilane and yielded 80% of the crude peptide containing approximately 4% of the pyroglutaminyl peptide. The crude peptides were precipitated by cold ether and separated from the pyroglutaminyl 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. Lyophylization 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-βNA, 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-naphthylamine under assay conditions. One unit is defined as the amount of QC catalyzing the formation of 1 μmol pGlu-βNA from H-Gln-βNA per minute under the described conditions.

The kinetic parameters for conversion of H-Gln-Gln-OH, H-Gln-NH_2 (both from Bachem), H-Gln-His-Pro-NH_2, and H-Gln-Tyr-Ala-OH by QC were determined spectrophotometrically using a continuous assay developed on basis of a previously described method (*24*) (manuscript in preparation).

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 using the Jasco secondary structure estimation program based on the method of Yang et al. (*25*).

For investigation of the structural changes of QC, fluorescence emission spectra were recorded in the wavelength range between 300 and 400 nm using the luminescence spectrometer LS 50 B (Perkin-Elmer). The excitation wavelength was 295 nm. Spectra were recorded at 100 nm/min using slit widths of 3 nm for excitation and 6 nm for emission.

The disulfide bond status was investigated by SDS−PAGE using deglycosylated QC. Samples were prepared under reducing (5% 2-mercaptoethanol) and nonreducing conditions as described previously (*26*). Electrophoresis was carried out in 15% polyacrylamide gels (Mini-Protean 3, BioRad), at constant voltage (200 V) for 2 h. Additionally, the determination of mercaptans in proteins by a dye reagent, Bis(3-carboxy-4-nitrophenyl)disulfide, was performed as described by Ellman (*27*).

MALDI-TOF mass spectrometry and deglycosylation assay. Matrix-assisted laser desorption/ionization mass spectrometry was carried out using the Hewlett-Packard G2025 LD-TOF System 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 the matrix solution, we used 2′,6′-dihydroxyacetophenone (DHAP, Aldrich)/diammonium hydrogen citrate (DAHC, Fluka), prepared by solving 30 mg DHAP and 44 mg DAHC in 1 mL acetonitrile/0.1% TFA in water (1/1, v/v). A small volume ($\sim$1 μl) of the matrix-analyte-mixture was transferred to a probe tip and immediately evaporated in a vacuum chamber (Hewlett-Packard G2024A sample prep accessory) to ensure rapid and homogeneous sample crystallization. For determination of the molecular mass of QC, bovine serum albumin was added as an internal standard. The deglycosylation experiments were performed after external calibration using bovine serum albumin. For the deglycosylation, 8 μg QC was treated with 4×10^{-5} (0.4 NEB units) units of endoglycosidase H_f (New England Biolabs) in 0.05 M sodium citrate, pH 5.5, at room temperature, without denaturation of the protein. Aliquots were removed at different times, the deglycosylation process was stopped by addition of matrix (1:1 v/v), and the respective mass spectra were evaluated.

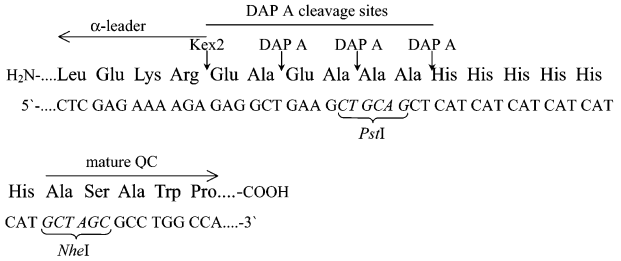

FIGURE 1: Partial sequences of the cDNA sense strand of the construct used for expression of QC in *P. pastoris* and its corresponding amino acid sequence. The cleavage sites of Kex2 and DAP A are indicated. The proteolytic processing results in a secreted, 6×histidine tagged QC, starting with alanine 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 *Pme*I 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 (OD_{600}), cell wet 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.

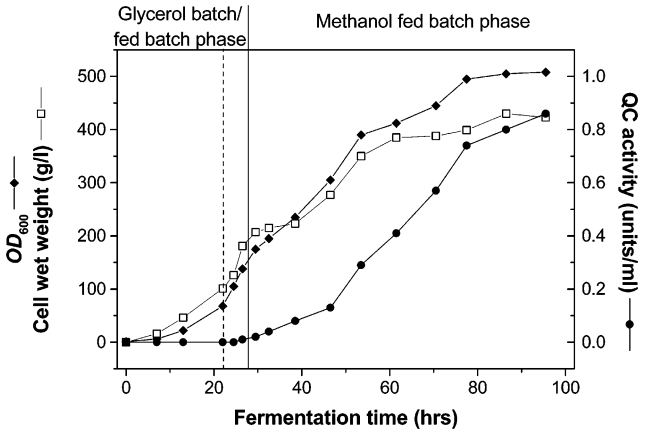

FIGURE 2: Time course of OD_{600}, cell wet weight and QC activity in fermentation of a recombinant strain of *P. pastoris* expressing human QC. The fermentation can be subdivided into three stages, the glycerol batch phase for about 24 h, followed by the glycerol fed batch phase for about 5 h, and finally by the methanol fed batch phase.

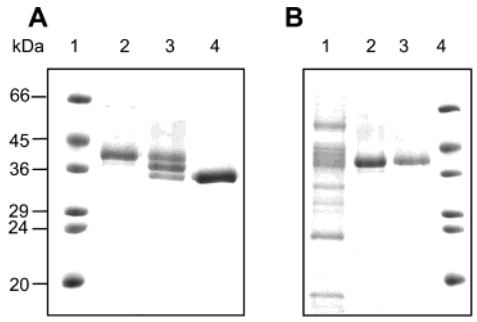

FIGURE 3: Characterization of human QC using SDS−PAGE. (A) SDS−PAGE analysis of QC-containing fractions of three different fermentations after IMAC. Lanes: 1, molecular mass standards (kDa); 2, 55 h fermentation; 3, after 75 h; 4, after 96 h. (B) SDS−PAGE illustrating the purification procedure of human QC. Lanes: 1, fermentation medium after expression; 2, the QC-containing fraction after IMAC; 3, purified QC after ion-exchange chromatography; 4, molecular mass standards. Electrophoresis was performed in 12% gels using reducing sample preparation as described elsewhere (*41*). Proteins were visualized by Coomassie staining.

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

Table 2: Purification Scheme of Recombinant Human QC Following Expression in *P. pastoris*

purification step	protein (mg)	QC activity (units)	specific activity (units/mg)	yield (%)
fermenation broth	393.2	362.8	0.9	100
immobilized metal ion affinity chromatography (IMAC)	41.6	256.8	6.2	71
ion-exchange chromatography (IEC)	4.1	76.9	18.7	21

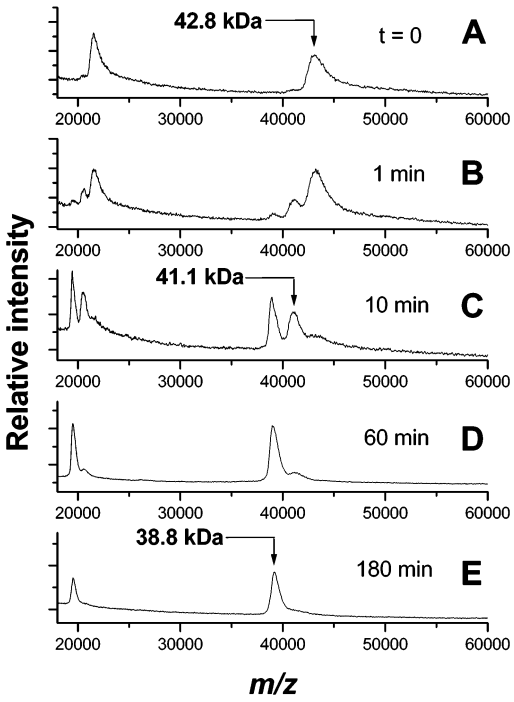

FIGURE 4: Deglycosylation of recombinant human QC, monitored by MALDI-TOF mass spectrometry. 8 μg of QC was treated with 4×10^{-5} units of endoglycosidase H_f in 0.05 M sodium citrate, pH 5.5, at room temperature. At the times indicated, samples were removed and diluted with matrix and the mass spectra recorded. The peak at 42.8 kDa corresponds to the double glycosylated QC. The peaks at 41.1 and 38.8 kDa represent the less glycosylated QC forms. The 2-fold charged proteins correspond to the peaks around 20 kDa.

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 H_f, 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 H_f. Therefore, post-translational modifications other than N-glycosylation are

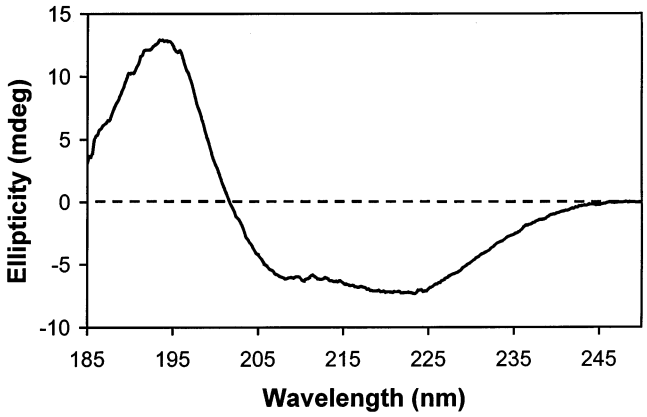

FIGURE 5: CD-spectroscopic analysis of the secondary structure of recombinant human QC. The protein was dissolved in 20 mM potassium phosphate buffer, pH 6.8. Estimation of the secondary structure revealed 47% α-helix, 14% β-sheet, and 14% β-turn content.

Table 3: Kinetic Parameters Determined for Human QC Expressed in *P. pastoris*[a]

QC substrate	Michaelis constant (μM)	turnover number (s^{-1})
H-Gln-Tyr-Ala-OH	101 ± 4	125 ± 1
H-Gln-His-Pro-NH_2	90 ± 4	83 ± 1
H-Gln-AMC	51 ± 3	5.4 ± 0.1
H-Gln-βNA	60 ± 6	18.8 ± 0.7
H-Gln-NH_2	409 ± 40	12.8 ± 0.5
H-Gln-Gln-OH	148 ± 5	20.7 ± 0.2

[a] Reactions were carried out at 30 °C in 0.05 M Tris/HCl, pH 8.0, containing 5 mM EDTA.

improbable during expression, and the N-terminus seems to be completely processed by Kex2 and DAP A, a frequent cause of inhomogeneities 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-NH_2, H-Gln-AMC, H-Gln-βNA, H-Gln-NH_2, and H-Gln-Gln-OH as substrates are listed in Table 3. Upon examination at 37 °C, the kinetic parameters K_m and k_{cat} 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 K_m values of 52 ± 5 μM and 43 ± 4 μM and k_{cat} 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,

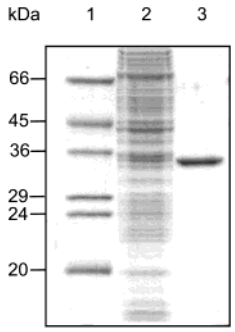

FIGURE 6: SDS−PAGE of the purification steps of human QC expressed in *E. coli*. Lanes: 1, molecular mass standards (kDa); 2, supernatant after lysis, 3, purified QC after IMAC. Electrophoresis was performed in 12% gels using reducing sample preparation as described elsewhere (*41*).

a K_m value of 0.64 mM and a k_{cat} of 50.9 min^{-1} were obtained for conversion of H-Gln-Gln-OH. Also, H-Gln-NH_2 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 K_m value of 62 ± 5 μM and a k_{cat} 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 K_m 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 H_f 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-

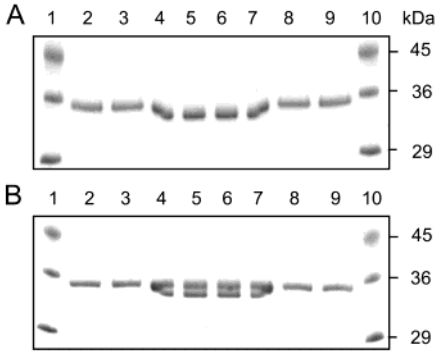

FIGURE 7: Disulfide-status of QC expressed in *P. pastoris* (A) and *E. coli* (B), examined using SDS−PAGE (*27*). Lanes: 1 and 10, molecular mass standards (kDa); 2, 3, 8, and 9, sample prepared under reducing conditions (5% β-mercaptoethanol); 4−7, sample prepared under nonreducing conditions. Electrophoresis was performed in 15% gels (6 × 8 cm) at constant voltage of 200V for 2 h and protein visualized by Coomassie staining. Due to diffusion of the reducing agent from lanes 3 and 8 into lanes 4 and 7, respectively, the band pattern was a mixed type.

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

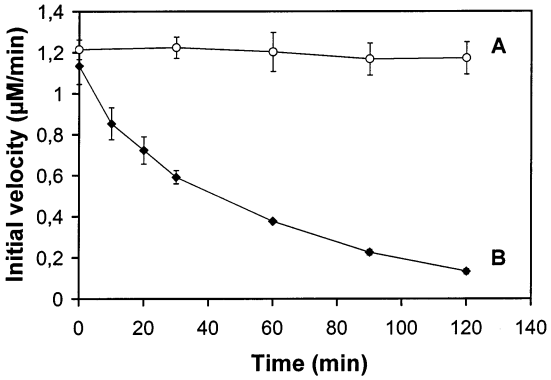

FIGURE 8: Inactivation of QC by 15 mM DTT (B). Reactions were carried out in 0.05 M potassium phosphate, pH 6.8, containing 0.3 M NaCl, and started by addition of 8 μg QC. At times indicated, samples were withdrawn and diluted 100-fold in 0.05 M Tris/HCl, pH 8.0, and the residual QC activity was determined using H-Gln-βNA as substrate. In absence of a reducing agent, enzyme activity remained constant (A).

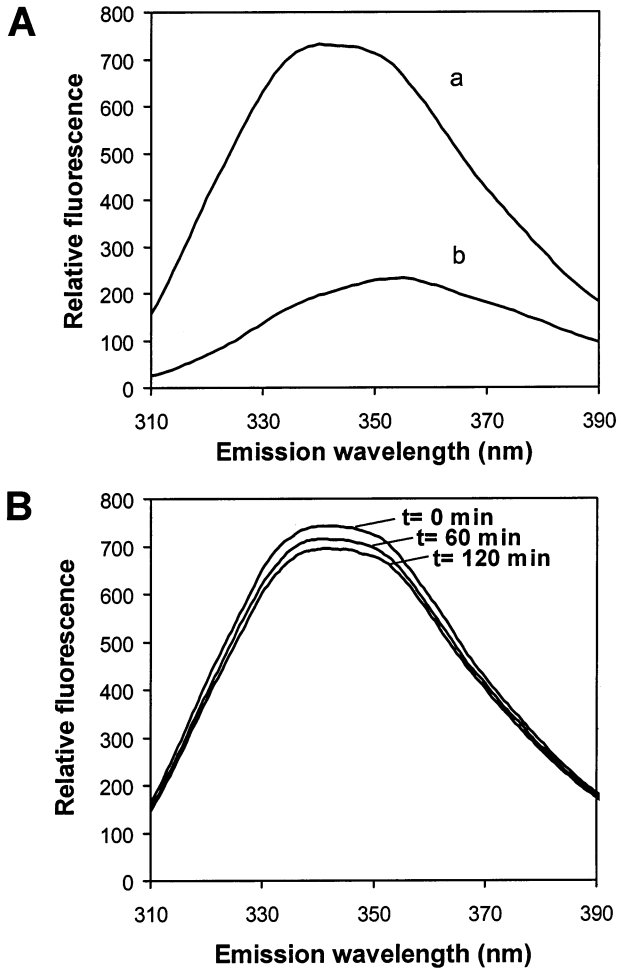

FIGURE 9: Fluorescence emission spectra of QC. (A) Spectra of 0.18 μM enzyme samples recorded in 0.05 M sodium phosphate, pH 6.8, containing 0.3 M NaCl in absence (a) and presence (b) of 6 M GdmCl after excitation at 295 nm. (B) Respective spectra recorded in the presence of 15 mM DTT at indicated times after addition of the reducing agent.

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 α-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 glycosylation was 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 $Man_{8-14}GlcNAc_2$, 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_f (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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