The soluble Y115E–Y117E variant of human glutaminyl cyclase is a valid target for X-ray and NMR screening of inhibitors against Alzheimer disease

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Recent developments in molecular pathology and genetics have allowed the identification of human glutaminyl cyclase (hQC) among the abnormal proteins involved in many neurodegenerative disorders. Difficulties in obtaining large quantities of pure protein may limit the use of crystallographic screening for drug development on this target. Site-directed mutagenesis experiments have led to the identification of some solvent-exposed residues that are absolutely critical to achieve increased solubility and to avoid precipitation of the enzyme in inclusion bodies when expressed in *Escherichia coli*. The designed variant Y115E–Y117E has been found to be able to provide large amounts of monodisperse, pure hQC from an *E. coli* expression system. To validate the use of the artificial construct as a target for large-scale X-ray and NMR screening campaigns in the search for new inhibitors of hQC, the X-ray crystal structures of the hQC Y115E–Y117E variant and of its adduct with the inhibitor PBD-150 were determined.

1. Introduction

Glutaminyl cyclase (QC) belongs to the class of transferases and catalyzes the formation of pyroglutamic acid (pGlu) from N-terminal glutaminyl or glutamyl precursors of several bioactive peptides and proteins. QC has been identified in animals, plants and bacterial sources (Castaldo *et al.*, 2013; Busby *et al.*, 1987; Huang, Liu & Wang, 2005; Messer, 1963). Owing to its potential involvement in the formation of pGlu-modified amyloid peptides, hQC is considered to be a valid drug target in Alzheimer disease (AD) and in the familial British and Danish dementias (Schilling *et al.*, 2008). A β peptide bearing an amino-terminal pyroglutamate at position 3 [A β N3(pE)] has been shown to be a major N-truncated, modified constituent of intracellular, extracellular and vascular A β deposits in AD brain tissue (Saido *et al.*, 1996; Harigaya *et al.*, 2000). A β N3(pE) shows a particular tendency to form a soluble oligomer that favours aggregation processes, also increasing the rate of aggregation of nonmodified peptides (Harigaya *et al.*, 2000).

The ability to characterize hQC through biochemical and biophysical assays and to use X-ray and NMR approaches to screen libraries of potential hQC inhibitors depends on the availability of large quantities of purified protein. To date, hQC has been expressed in *Drosophila* insect cells (Booth *et al.*, 2003), in the yeast *Pichia pastoris* (Ruiz-Carrillo *et al.*,

Table 1

Human glutamyl cyclase hQC-2X production information.

Source organism	<i>Homo sapiens sapiens</i>
DNA source	cDNA encoding human QC (Ala33–Leu361)
Forward primer	5'-CTTGAGTCAGACACCCGAAGGGGAACGGTCT-TTCTCAAATATC-3'
Reverse primer	5'-GATATTTGAGAAAGACCGTTCCTTCGGGT-GTCTGACTCAAG-3'
Cloning vector	pQE80L
Expression vector	pQE-80L-hQC (Ala33–Leu361)
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced	ASAWPEEKNYHQPAILNSSLARQIAEGTSISEMW-QNDLQPLLIERYPGSPGSAARQHMQRIQRL-QADWVLEIDTFLSQTPEGERSFSNIISTLNPT-AKRHLVLACHYDSKYFSHWNRVFVGATDSAV-PCAMMLELARAALDKLLSLKTVDSPDLQLQ-LIFFDGEEAFLHWSQDSLYGRHLAAKMAST-PHPPGARGTSQLHGMDDLVLLDLIGAPNPTFP-NFFPNSARWFERLQATIEHELHELGLLDHSLE-GRYFQNYSGGVIQDDHIPFLRRGVPLHLIP-SPFPEVWHTMDNEENLDESTIDNLNKILQVF-VLEYLHL

2011) and in *Escherichia coli* as a thioredoxin-fusion protein (Huang, Liu & Wang, 2005). These expression systems have drawbacks in terms of cost, ease of production or low yield. Consequently, a general method that is able to provide milligrams of soluble hQC is of great practical importance (Baneyx, 1999). A soluble variant of hQC has been designed in order to overcome the tendency of recombinant bacterial hQC to be expressed as inclusion bodies (Castaldo *et al.*, 2013). This goal has been reached by substituting two tyrosine residues on the surface of hQC with glutamates to obtain a soluble but still active form of the protein (Castaldo *et al.*, 2013).

Here, we provide evidence that the designed hQC variant with higher solubility is a valid target for large-scale X-ray crystallographic screening of ligands.

2. Materials and methods

2.1. Macromolecule production

The gene-coding sequence for human QC double mutant Y115E–Y117E (hereafter referred to as hQC-2X) was cloned in pQE-80L expression vector, which includes a noncleavable histidine tag. The expression plasmid was introduced into *E. coli* strain BL21 (DE3) by thermal shock (see Table 1).

The bacterial culture was grown at 37°C in SB medium supplemented with 100 mg l⁻¹ ampicillin. Protein overexpression was induced with 0.1 mM IPTG when the cell density reached an OD₆₀₀ of 0.6–0.8 and the culture was incubated at 24°C for 48 h.

After 2 d, the cells were harvested by centrifugation (4000 rev min⁻¹ for 15 min), resuspended in lysis buffer (50 mM Tris–HCl pH 8.5, 150 mM NaCl, 20 mM imidazole) and disrupted by sonication.

The supernatant of the resulting crude extract was collected by centrifugation and further purified by nickel-affinity chromatography similar to the previously described method (Castaldo *et al.*, 2013).

Table 2

Crystallization.

Method	Sitting-drop vapour diffusion
Plate type	24-well plates
Temperature (K)	277.15
Protein concentration (mg ml ⁻¹)	8
Buffer composition of protein solution	100 mM Tris–HCl pH 7.5, 0.15 M NaCl
Composition of reservoir solution	0.1 M MES buffer pH 6.5, 0.2–0.4 M ammonium sulfate
Volume and ratio of drop	6 µl, 1:1 ratio
Volume of reservoir (µl)	600

The yield of purified protein was around 35 mg per litre of bacterial culture. We repeated the production of hQC-2X in the cheaper LB medium using the same procedure, obtaining a yield of 25 mg of pure enzyme per litre of culture. Hence, hQC-2X can be produced in quite high yield using SB or LB medium.

2.2. Crystallization

In earlier studies employing enzyme from a different source, rhombohedral crystals (space group *H*32) of the native enzyme were obtained using 0.1 M MES buffer pH 6.5, 1.6 M ammonium sulfate as the precipitant solution (Huang, Liu & Wang, 2005). Attempts to crystallize the hQC-2X variant in conditions similar to those for the native enzyme were unsuccessful.

Crystals of hQC-2X were obtained using the sitting-drop vapour-diffusion technique (Benvenuti & Mangani, 2007). Drops were prepared by mixing equal volumes (3 µl) of 8 mg ml⁻¹ hQC-2X in 0.1 M Tris–HCl pH 7.5, 0.15 M NaCl with a precipitant solution composed of 0.2–0.4 M ammonium sulfate, 0.1 M MES pH 6.5 (Table 2). The different crystallization condition of hQC-2X compared with those for the native enzyme can possibly be attributed to the changed surface properties of our variant, which cause a different quaternary assembly of the molecules as indicated by the different space group and packing (see §3).

Drops were allowed to equilibrate at 277.15 K over wells containing 600 µl precipitant solution. Crystals suitable for diffraction appeared within one week.

Crystals of hQC-2X in complex with the PBD-150 inhibitor ([1-(3,4-dimethoxyphenyl)-3-[3-(1*H*-imidazol-1-yl)propyl]thio-

Figure 1

Chemical structure of 1-(3,4-dimethoxyphenyl)-3-[3-(1*H*-imidazol-1-yl)propyl]thiourea (PBD-150).

urea; $C_{15}H_{20}N_4O_2S$; Fig. 1) were obtained by soaking the enzyme crystals for 2 h in crystallization buffer containing 15 mM PBD-150 dissolved in 1,4-dioxane.

Before data collection, the crystals were transferred into a cryoprotectant solution consisting of 20% ethylene glycol and 80% precipitant solution and flash-cooled in liquid nitrogen.

2.3. Data collection, processing, structure solution and refinement

hQC-2X data were collected on beamline I04-1 at the Diamond Light Source (DLS) using a Pilatus 2M detector at 100 K. Data were integrated using *MOSFLM* v.7.0.4 (Leslie, 2006) and scaled with *SCALA* (Evans, 2006) from the *CCP4* suite (Winn *et al.*, 2011) (Table 3).

Structures were solved by molecular replacement using *MOLREP* (Vagin & Teplyakov, 2010) with a subunit of human glutaminyl cyclase (PDB entry 2afm; Huang, Liu, Cheng *et al.*, 2005), excluding nonprotein atoms and water molecules, as a search model.

The position of the zinc ion was verified by inspection of the anomalous difference Fourier map obtained from data collected at the high-energy remote from the Zn *K* edge (see Table 3).

The presence of the mutations, Y115E–Y117E, was verified using $F_o - F_c$ and $2F_o - F_c$ electron-density maps after molecular-replacement phasing using the native enzyme in which the two mutated residues had been deleted as a model.

The structure was refined with *REFMAC* (Murshudov *et al.*, 2011). The refinement protocol consisted of a sequence of iterative manual rebuilding of the model and maximum-likelihood refinement (Table 4).

Manual rebuilding and modelling of the missing atoms into the electron density was performed with *Coot* (Emsley & Cowtan, 2004).

The final model was inspected manually and checked with *Coot* and *PROCHECK* (Laskowski *et al.*, 1993).

3. Results and discussion

3.1. Crystal structure of the hQC double mutant Y115E–Y117E (hQC-2X)

The crystal structure of the double mutant hQC-2X determined by X-ray crystallography to 2.10 Å resolution in the monoclinic space group *C2* shows an asymmetric unit containing three independent molecules (Fig. 2) which adopt the same conformation and are almost identical within experimental error (pairwise root-mean-square deviation values in the range 0.14–0.16 Å). Analysis of the quaternary structure of the enzyme by *PISA* (Krissinel & Henrick, 2007; http://www.ebi.ac.uk/pdbe/prot_int/pistart.html) indicates that both the hQC-2X trimer and the dimer of trimers observed in the crystal packing might be stable in solution. However, hQC structures obtained from other sources show a different arrangement of subunits, indicating that the trimeric or hexameric quaternary structures are not physiological.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

	hQC-2X	hQC-2X–PBD-150
Diffraction source	I04-1, DLS	I04-1, DLS
Wavelength (Å)	0.979	0.979
Temperature (K)	100	100
Detector	Pilatus 2M	Pilatus 2M
Space group	<i>C2</i>	<i>C2</i>
<i>a</i> , <i>b</i> , <i>c</i> (Å)	86.38, 149.63, 96.30	86.43, 149.54, 96.21
β (°)	96.7	96.82
Mosaicity (°)	0.52	0.70
Resolution range (Å)	33.0–2.10 (2.21–2.10)	33.0–1.95 (2.06–1.95)
Total No. of reflections	154214 (22314)	257040 (36754)
No. of unique reflections	66975 (9807)	87147 (12723)
Completeness (%)	95.0 (95.3)	99.0 (98.9)
Multiplicity	2.3 (2.3)	2.9 (2.9)
$\langle I/\sigma(I) \rangle$	5.1 (2.7)	6.0 (2.8)
R_{meas} (%)	18.4 (42.7)	17.1 (36.7)
Overall <i>B</i> factor from Wilson plot (Å ²)	9.68	9.41

Table 4

Structure solution and refinement.

Values in parentheses are for the outer shell.

	hQC-2X	hQC-2X–PBD-150
Resolution range (Å)	74.81–2.10 (2.16–2.10)	74.77–1.95 (2.00–1.95)
No. of reflections, working set	62069 (4734)	82798 (6094)
No. of reflections, test set	4249 (253)	4184 (330)
Final R_{cryst} (%)	21.1 (21.5)	16.4 (21.4)
Final R_{free} (%)	25.4 (26.9)	21.1 (25.9)
Cruickshank DPI	0.2637	0.1473
No. of non-H atoms		
Total	8775	9269
Protein atoms	7758	7793
Ion atoms		
Zinc	3	3
Sulfate	15	25
Chloride	0	1
No. of ethylene glycol molecules	15	20
No. of ethylene glycol atoms	60	80
No. of ligand molecules	0	3
No. of ligand atoms	0	66
No. of waters	939	1301
R.m.s. deviations		
Bonds (Å)	0.016	0.018
Angles (°)	1.771	1.912
Average <i>B</i> factor (Å ²)	13.99	14.0
Ramachandran plot		
Most favoured (%)	97.2	96.7
Allowed (%)	2.8	3.3

Figs. 2 and 3 show the crystal packing of native hQC and of our variant, respectively. Comparison of Figs. 2(*a*) and 3(*a*), which show views along the respective crystallographic twofold axes of the *H32* and *C2* cells, show the different intersubunit arrangements of wild-type hQC and hQC-2X owing to intersubunit interactions that involve different portions of the molecules. In the case of wild-type hQC three dimers are arranged along the crystallographic threefold axis (Fig. 2*b*), with the perpendicular twofold axis relating subunits belonging to different dimers (Fig. 2*a*). Only the main-chain NH of the Tyr115 and Tyr117 residues are involved in intersubunit hydrogen bonds to the carbonyl group of Pro114 of a

facing dimer (Fig. 2*a*). Fig. 3(*a*) shows the packing of the hQC-2X trimers viewed along the crystallographic twofold axis. The interface of two subunits differs from that of wild-type hQC and involves hydrogen bonds between the side chain of Glu115 and the side chain of Arg233. Fig. 3(*b*) shows a view of the packing perpendicular to the twofold axis, revealing that a noncrystallographic pseudo-threefold axis is also maintained in the hQC-2X crystals.

Figure 2
Crystal packing of the wild-type hQC structure. (*a*) View along the twofold axis of the *H32* cell. (*b*) View along the threefold axis of the *H32* cell. The three dimers are shown as green, red and violet ribbons. The Pro114, Arg233, Tyr115 and Tyr117 residues are shown as yellow and cyan sticks, respectively.

The tertiary structure of hQC-2X has the mixed α/β -hydrolase fold with an open-sandwich topology as described previously (Huang, Liu, Cheng *et al.*, 2005), which is superimposable on the native enzyme (PDB entry 2afm) with r.m.s.d. values in the range 0.40–0.44 Å (Fig. 4).

The active site of hQC-2X is located in the random-coil region (Fig. 4), where the catalytic zinc ion is coordinated by

Figure 3
Crystal packing of the hQC-2X structure. (*a*) View along the twofold axis of the *C2* cell. (*b*) View perpendicular to the twofold axis showing the pseudo-threefold symmetry of the hQC-2X dimer of trimers. The Pro114, Arg233, Glu115 and Glu117 residues are shown as yellow and cyan sticks, respectively.

Asp159 OD2, Glu202 OE2, His330 NE2 and a water molecule (W406) that completes the tetrahedral coordination of the metal ion (Fig. 5a).

No relevant structural variations are present in the active site with respect to the wild-type enzyme. Additionally, in agreement with the wild-type hQC structure, the *cis* conformation of the peptide bond between Asp159, involved in the coordination of the catalytic zinc, and Ser160 is maintained in our double mutant and is stabilized by a hydrogen-bonding network involving Asp248. The residue Trp207, which closes the catalytic site of the enzyme, forming a wall, assumes a different conformation to that observed in the wild-type structure. Namely, the indole ring is flipped by 180°, as observed in other previously published hQC structures (PDB entries 3si0 and 3pbb; Ruiz-Carrillo *et al.*, 2011; Huang *et al.*, 2011). The different orientation of the Trp207 side chain is not correlated with the binding of the inhibitor and does not affect the orientation of the loop connecting the $\alpha 4$ and $\beta 7$ strands; it is a clear indicator of the flexibility of the residue, which is reasonably linked to the different conformation of His206.

The mutated residues Tyr115 and Tyr117 have been completely rebuilt in all three subunits according to the electron density (Fig. 5b). Although strongly affecting the protein solubility, these mutations do not influence the orientation of the loop connecting the $\beta 1$ and $\beta 2$ strands in which they are located, which overlaps well with the corresponding region of the native protein. The selective superimposition of the C α atoms of residues 111–118 of the mutant with the same residues in the native hQC structure (PDB entry 2afm) gives an r.m.s.d. value of 0.18 Å.

Figure 4
Cartoon representation of superimposition of the unligated hQC-2X crystal structure (pale cyan) with the crystal structure of wild-type hQC (green; PDB entry 2afm; Huang, Liu, Cheng *et al.*, 2005). The zinc ion is represented as a magenta sphere.

3.2. Crystal structure of the hQC double mutant Y115E–Y117E in complex with PBD-150

Crystals of the hQC-2X variant in complex with PBD-150 belong to the same monoclinic space group, *C2*, as the ligand-free variant, with three molecules per asymmetric unit. The structure has been determined to 1.95 Å resolution.

The inhibitor molecule is bound to the catalytic cavity of all three subunits of hQC-2X and its binding does not affect the general fold of the protein (r.m.s.d. value of 0.19 Å with respect to the ligand-free variant).

The PBD-150 bound to hQC-2X adopts the same pose as in the wild-type complex (PDB entry 3pbb; Huang *et al.*, 2011). As shown in Fig. 6, the enzyme–inhibitor interactions inside

Figure 5
(a) Electron-density map (blue wire; $2F_o - F_c$ map contoured at 1.25σ) showing the active site of the hQC-2X variant. The Zn^{2+} ion (grey sphere) is identified by the anomalous difference map (red wire) superimposed on the $2F_o - F_c$ map. (b) Electron-density map (blue wire; $2F_o - F_c$ map contoured at 1.25σ) showing the Glu residues at positions 115 and 117 (blue sticks) in the hQC-2X variant.

Figure 6

Key interactions of PBD-150 (magenta sticks) in the active site of hQC-2X. The zinc ion is shown as a grey sphere. The $2F_o - F_c$ map (blue wire; contoured at 1.25σ) and the anomalous difference map (red wire) are superimposed.

the catalytic pocket are exactly the same: the N atom of the imidazole ring of the inhibitor interacts with the catalytic zinc ion to complete the tetrahedral coordination of the metal, replacing the water molecule observed in ligand-free hQC-2X. The rest of the inhibitor molecule points away from the catalytic site. The S atom of the PBD-150 thioketone moiety is hydrogen-bonded to the backbone NH of Gln304; the aromatic terminal portion of the molecule, which is more exposed to solvent, interacts with the aromatic side-chain ring of Phe325 through a displaced π - π stacking, and the methoxy O atom makes a water-mediated interaction with the backbone NH of the same residue (Fig. 6).

Fig. 7 shows the positions of the bound sulfate anions with respect to the hQC active site in ligand-free hQC-2X (Fig. 7*a*), PBD150-inhibited hQC-2X (Fig. 7*b*) and wild-type hQC (Fig. 7*c*). In contrast to the wild-type hQC crystal, in hQC-2X the sulfate anions bind away from the active-site cavity, leaving free access to the cavity.

Comparison of the structures of hQC-2X and the hQC-2X-PBD-150 complex with the analogous wild-type hQC structures demonstrates that the mutations inserted into hQC in order to obtain a more soluble construct do not influence the binding mode of the inhibitors and fully validates the use of this mutant in crystallographic and NMR screening of inhibitors. hQC-2X provides a low-cost, easy-to-produce hQC variant that will be useful to foster the discovery of novel inhibitors of the glutamyl cyclase enzyme.

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Figure 7

(*a*) The binding site of the sulfate anion (ball-and-stick model) in ligand-free hQC-2X. (*b*) The binding site of the sulfate anion in PBD150-bound hQC-2X remains the same. (*c*) The sulfate anion bound to wild-type hQC differs from those reported for hQC-2X and is much closer to the active site.

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References

- Baneyx, F. (1999). *Curr. Opin. Biotechnol.* **10**, 411–421.
- Benvenuti, M. & Mangani, S. (2007). *Nature Protoc.* **2**, 1633–1651.
- Booth, R. E., Misquitta, S. A. & Bateman, R. C. Jr (2003). *Protein Expr. Purif.* **32**, 141–146.
- Busby, W. H. J., Quackenbush, G. E., Humm, J., Youngblood, W. W. & Kizer, J. S. (1987). *J. Biol. Chem.* **262**, 8532–8536.
- Castaldo, C., Ciambellotti, S., de Pablo-Latorre, R., Lalli, D., Porcari, V. & Turano, P. (2013). *PLoS One*, **8**, e71657.
- Emsley, P. & Cowtan, K. (2004). *Acta Cryst. D* **60**, 2126–2132.
- Evans, P. (2006). *Acta Cryst. D* **62**, 72–82.
- Harigaya, Y., Saido, T. C., Eckman, C. B., Prada, C. M., Shoji, M. & Younkin, S. G. (2000). *Biochem. Biophys. Res. Commun.* **276**, 422–427.
- Huang, K.-F., Liaw, S.-S., Huang, W.-L., Chia, C.-Y., Lo, Y.-C., Chen, Y.-L. & Wang, A. H.-J. (2011). *J. Biol. Chem.* **286**, 12439–12449.
- Huang, K.-F., Liu, Y.-L., Cheng, W.-J., Ko, T.-P. & Wang, A. H.-J. (2005). *Proc. Natl Acad. Sci. USA*, **102**, 13117–13122.
- Huang, K.-F., Liu, Y.-L. & Wang, A. H.-J. (2005). *Protein Expr. Purif.* **43**, 65–72.
- Krissinel, E. & Henrick, K. (2007). *J. Mol. Biol.* **372**, 774–797.
- Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993). *J. Appl. Cryst.* **26**, 283–291.
- Leslie, A. G. W. (2006). *Acta Cryst. D* **62**, 48–57.
- Messer, M. (1963). *Nature (London)*, **197**, 1299.
- Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst. D* **67**, 355–367.
- Ruiz-Carrillo, D., Koch, B., Parthier, C., Wermann, M., Dambe, T., Buchholz, T., Ludwig, H.-H., Heiser, U., Rahfeld, J.-U., Stubbs, M. T., Schilling, S. & Demuth, H.-U. (2011). *Biochemistry*, **50**, 6280–6288.
- Saido, T. C., Yamao-Harigaya, W., Iwatsubo, T. & Kawashima, S. (1996). *Neurosci. Lett.* **215**, 173–176.
- Schilling, S. *et al.* (2008). *Nature Med.* **14**, 1106–1111.
- Vagin, A. & Teplyakov, A. (2010). *Acta Cryst. D* **66**, 22–25.
- Winn, M. D. *et al.* (2011). *Acta Cryst. D* **67**, 235–242.