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Role of the distal hinge region of C1-inhibitor in the regulation of C1s activity

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Abstract A synthetic peptide corresponding to residues 448–459 of C1-inhibitor (C1-inh) binds to C1s, is a non-competitive inhibitor of C1s activity and prevents formation of an SDS-stable C1s–C1-inh complex. Substitutions of residues Q452, Q453 or F455 in this peptide resulted in loss of C1s binding and inhibitory activity of the peptide. NMR analysis of the peptide showed an area of well-defined structure from E450 to F455. The side chains of Q452, Q453 and Q455 were exposed to the solvent and therefore available for C1s binding. The defined structure in the peptide is compatible with our computer model of the serpin domain of C1-inh.

Key words: Human; Complement; Biochemistry

1. Introduction

Human C1-inhibitor (C1-inh) is a single polypeptide chain glycoprotein of 478 residues as predicted from cDNA sequencing [1,2] with a calculated polypeptide molecular weight of 52.8 kDa. On SDS-PAGE it appears as a glycosylated protein of apparent MW 115 kDa and 105 kDa under non-reducing and reducing conditions respectively. C1-inh belongs to the superfamily of serine protease inhibitors (serpins), and inactivates proteases of the contact phase (Factor XIIa) [3,4], coagulation Factor XIa [3], complement (activated C1, C1r and C1s) [5], tissue-type plasminogen activators [6] and kallikrein [5,7]. C1-inh acts on the C1 molecule both by binding to proenzymic C1r and C1s to prevent autoactivation and by binding to the activated C1r and C1s proteases in C1, which results in dissociation of C1r and C1s from the C1 complex, in the form of a C1-inh–C1r–C1s–C1-inh tetramer [8]. During interaction of C1-inh with activated C1r or C1s, the active site serine of the protease attacks the peptide bond between Arg^{444} and Thr^{445} of C1-inh and an SDS-resistant enzyme–inhibitor complex is formed [9]. This complex is an enzyme–substrate or enzyme–product acyl intermediate, which is not subsequently hydrolysed. Formation of the complex indicates that the product of C1s proteolytic activity, cleaved C1-inh, is likely to have a strong secundary interaction with the protease, which prevents dissociation of the complex.

Recent studies on the pathogenesis of acquired C1-inh deficiency in humans have shown that circulating autoantibodies are present in the vast majority of patients [10] and C1-inh is usually present in the cleaved (96 kDa) functionally inactive form [11]. The C1-inh deficiency leads to the disease acquired or autoimmune angiooedema. In six such patients the anti-C1-inh autoantibody recognised an epitope in the distal hinge region [12] and converted C1-inh from an inhibitory pseudo-substrate to a substrate [13]. The autoantibodies from these six patients bound to a synthetic peptide, corresponding to residues 448–459, derived from the distal hinge region of C1-inh and the epitope recognised included residues Gln^{452}, Gln^{453}, Pro^{454} and Phe^{455} [12]. This peptide was subsequently shown to bind to C1s. Pre-incubation of C1s with the peptide prior to the addition of C1-inh prevented the formation of SDS-resistant C1s–C1-inh complexes and C1-inh was cleaved into a 96 kDa form. These data led us to conclude that the distal hinge region of C1-inh contained a binding site for C1s which was (a) distinct from the site of proteolysis by C1s and (b) essential for the formation of the SDS-resistant C1s–C1-inh complex [14].

The present study is concerned with defining the interaction of individual residues of C1-inh with C1s at the secondary interaction site. The data presented show that the secondary interaction site of C1-inh is required for regulating C1s activity and that sequence alterations in the distal hinge region of C1-inh will alter the secondary binding site so that the binding of C1s to C1-inh is diminished. We also provide direct NMR evidence to show that the conformation of the residues Gln^{452} to Phe^{455} in the peptide 448–459 is similar to that predicted for the secondary interaction site in the intact C1-inh molecule.

2. Materials and methods

2.1. Synthetic peptides

Two peptides, one spanning the proximal hinge region (residues 428–440, peptide 1, TETGVEAAAASAI) and the other spanning the distal hinge region (residues 448–459, peptide 2, VFEVQQPFFVL) of C1-inh and 13 altered peptides derived from peptide 2 (E450L, E450Q, E450N, Q452E, Q452D, Q453E, Q453D, Q453L, Q452L/Q453L, Q452D/Q453D, F455L, F456L and F455L/F457L), were obtained from Alta Biosciences (Department of Biochemistry, University of Birmingham, UK).

Activated C1s [15] and C1-inh [16] were purified by standard techniques. Binding of C1s to solid-phase synthetic peptides was determined by ELISA [14]. Briefly plates (Falcon, Becton Dickinson Labware Company, USA) were coated with peptides at 100 μg/ml and C1s was used at 10 μg/ml. Bound C1s was detected using anti-human C1s (Incstar Ltd., Wokingham, UK) followed by HRP-conjugated rabbit antigoat IgG (Incstar).

Effects of synthetic peptides on C1s enzymic activity were analysed using the chromogenic substrate propionyl L-lysyl L-arginine *p*-nitroanilide hydroacetate (AcOH–C_2H_5CO–Lys(e-Cbo)–Gly–Arg–pNA) (Immuno R Ltd., Heidelberg, Germany) dissolved in 50 mM Tris-HCl, pH 8.5, containing 25 mM NaCl [14].

NMR spectroscopy on peptide 2 was undertaken using a Varian

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Unity 500 MHz NMR spectrometer at 25°C. The complete assignments of the 2D spectra were based on published methods [17,18]. Complete assignments of the ^{1}H-NMR spectra were achieved by following the connectivity along the entire molecule with the combination of TOCSY and NOESY spectra [19,22]. Full details are given in the legend to Fig. 3.

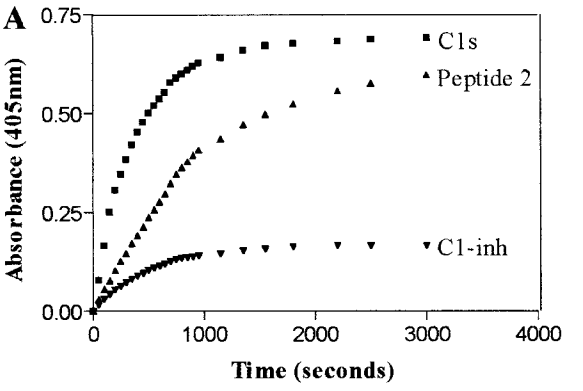
The secondary C1s binding site on C1-inh was modelled with Silicon Graphics using ovalbumin co-ordinates [23] and confirmed with antithrombin III data [24] (provided by Professor Robin Carrell, Department of Haematology, Cambridge University, UK). Residue replacements were made according to serpin sequence alignments.

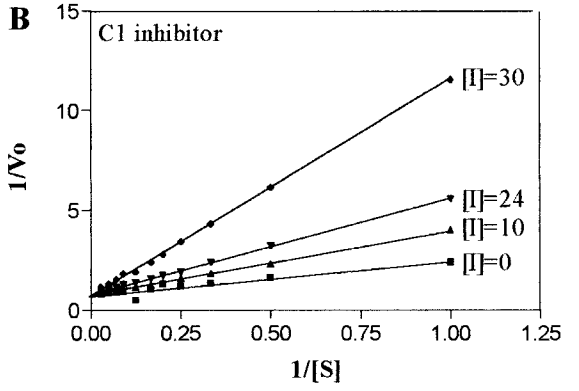
3. Results and discussion

Fig. 1A shows progress curves for the inhibition of C1s activity by C1-inh and peptide 2 (both at 25 nM). In the presence of C1-inh, the extent of substrate hydrolysis reached a plateau showing that C1-inh binds tightly to C1s. In contrast, peptide 2 slowed the initial rate of substrate hydrolysis, but by the end of the incubation period hydrolysis was approaching completion. Thus peptide 2 is not an irreversible inhibitor of C1s activity. Peptide 1 did not affect C1s activity (data not shown). The differences in the inhibitory activities of C1-inh and peptide 2 were explored further in second series of experiments in which different substrate concentrations (1–20 μM), and concentrations of C1-inh and peptide were used with a single fixed concentration of C1s (24 nM). Lineweaver-Burk plots confirmed that the patterns of inhibition of C1s by C1-inh and peptide 2 were different (Fig. 1B), with C1-inh behaving as a competitive inhibitor while peptide 2 behaved non-competitively. As non-competitive inhibitors are able to bind to the enzyme-substrate complex, they must bind to a site which is distinct from the enzyme cleft. This finding is consistent with our earlier observation that peptide 2 binds to C1s and, when pre-incubated with C1s, caused C1-inh to behave as a substrate rather than an inhibitor [14]. We therefore hypothesised that the distal hinge region of C1-inh contained a secondary binding site for C1s and that the interaction of this site with C1s was essential for the formation of SDS-stable C1s–C1-inh complexes [14]. Peptide 2 would therefore act by binding to C1s, and preventing C1-inh from binding to the same site.

In order to determine which residues in peptide 2 contribute to the secondary binding site for C1s a series of peptide 2 derivatives was investigated for C1s binding and C1s inhibitory activities. The substitutions Q452E, Q452D, Q453E, Q453D, Q453L and F455L showed marked reduction in C1s binding (Fig. 2, top) and inhibition of C1s activity (Fig. 2, bottom). Substitutions of L or Q for E at position 450 did not affect C1s binding or activity, indicating that this residue does not contribute significantly to the secondary binding site for C1s. These data indicate that the residues Q452, Q453 and F455 of C1-inh are involved in the binding of C1s at the secondary binding site and, in peptide 2, are involved in the regulation of C1s activity.

The structural basis for the interaction of peptide 2 with C1s was investigated by NMR spectrometry. Complete assign-





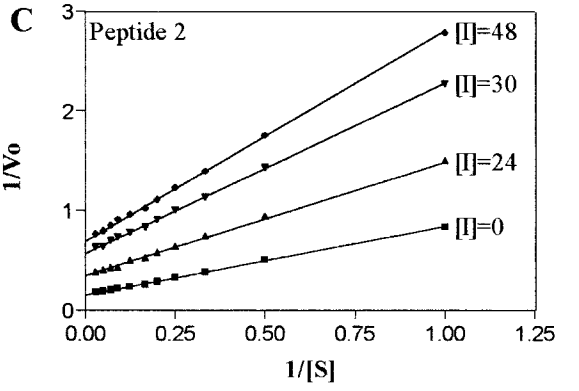
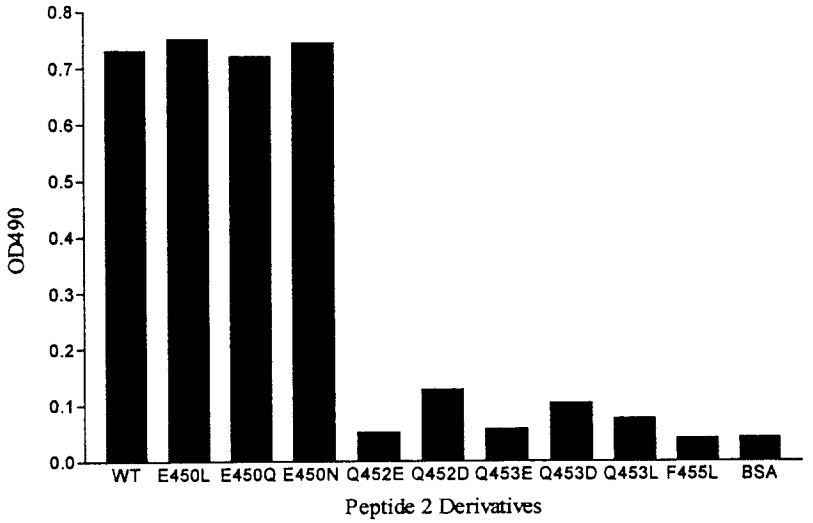


Fig. 1. A: Kinetic assays for the inhibition of C1s activity by C1-inh and peptide 2. C1s and C1-inh were dialysed against 10 mM Tris-HCl, pH 8.0, containing 100 mM NaCl and 1 mM EDTA. The chromogen was dissolved in 50 mM Tris-HCl, pH 8.0, containing 25 mM NaCl to provide a stock solution with a concentration of 36 μM. All final reaction volumes were adjusted to 800 μl using 10 mM Tris-HCl, pH 8.0, containing 100 mM NaCl. Progress curves were performed with concentrations of C1s, C1-inh and peptide each at 25 nM and chromogen at a concentration of 15 μM. Immediately after C1s, C1-inh or peptides and chromogen were mixed, the absorbance changes were recorded at 450 nM for 50 cycles at 60 s intervals using a Perkin Elmer spectrophotometer. B,C: The concentration of C1s was kept at 24 nM in each reaction. C1-inh was added to final concentrations of 10, 24 or 30 nM while peptide 2 was added to final concentrations of 24, 30 or 48 nM. The mixing of C1s and inhibitor was followed immediately by the addition of a series of different concentrations of chromogen (final concentration 1–20 μM in 20 reaction mixtures) for each concentration of C1-inh or peptide. The absorbance changes were then recorded automatically at 405 nm. The initial reaction velocity (V_0) for each reaction was calculated from data recorded for 20 cycles at 20 s intervals. B: C1-inh; C: peptide 2.



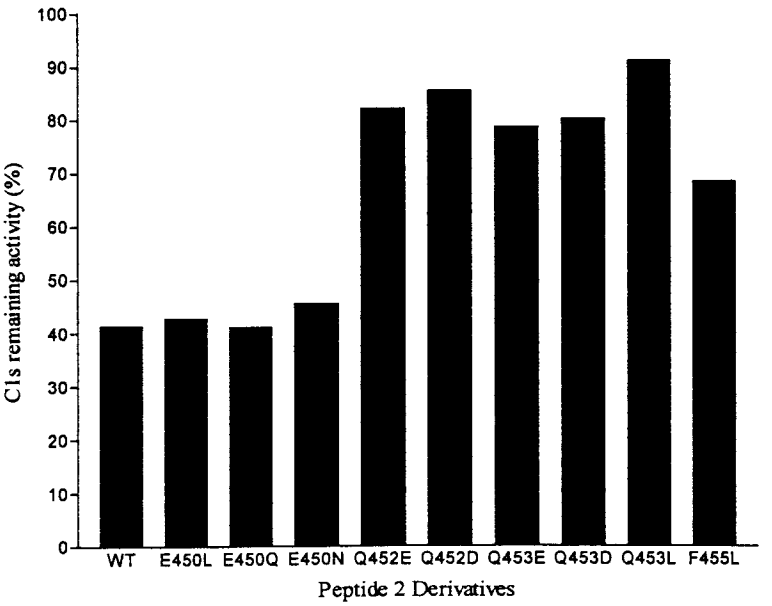


Fig. 2. Top: Effect of sequence variation in peptide 2 on binding to C1s and inhibition of C1s enzymic activity. Peptides were dissolved in 20 mM Tris-HCl, pH 7.6, 150 mM NaCl and 10% (v/v) DMSO and then diluted in coating buffer (15 mM Na_2CO_3, 3.5 mM $NaHCO_3$, pH 9.6) to final peptide concentrations of 100 μg/ml. ELISA plates were coated by incubating 100 μl of solution/well overnight at 4°C. Non-specific binding sites were blocked by incubation with 0.1% gelatin in PBS. C1s (10 μg/ml in PBS) was added and incubated at 37°C for 1 h followed by addition of goat anti-human C1s (1:2000 dilution in PBS) at room temperature for a further 2 h and then anti-goat immunoglobulin, conjugated with horseradish peroxidase (HRP; 1:4000). Incubation was carried out at room temperature for a further 2 h. The plate was washed 3 times in PBS, containing 0.2% (v/v) Tween 20, between each step. The plate was developed using OPD (2.1% (w/v) *o*-phenylenediamine dihydrochloride in 22 mM sodium citrate, 56 mM Na_2HPO_4, pH 5.0, and 5% (v/v) of fresh 30% H_2O_2). The reaction was stopped by addition of 4 M H_2SO_4 and the absorbance was read at 490 nm. Bottom: Kinetic assay for the effect of peptide 2 and its derivatives on C1s activity. A standard inhibition curve was constructed using a fixed concentration of C1s and synthetic peptide (both at 24 nM). This series of reactions was pre-incubated at 37°C for 30 min before the addition of the chromogen to a final concentration of 10 μM. The absorbance changes at 405 nm were then recorded at 20 s intervals for 6 min. The percentage of residual C1s activity was calculated using the equation

$$(1-\frac{x-y}{x})\times 100$$

where x = slope for C1s alone and y = slope of C1s in presence of peptide.

ments of the ^{1}H-NMR spectra were achieved by following the connectivity along the entire length of peptide 2 with the combination of TOCSY and NOESY spectra. Molecular modelling revealed a well-defined structure from E450 to

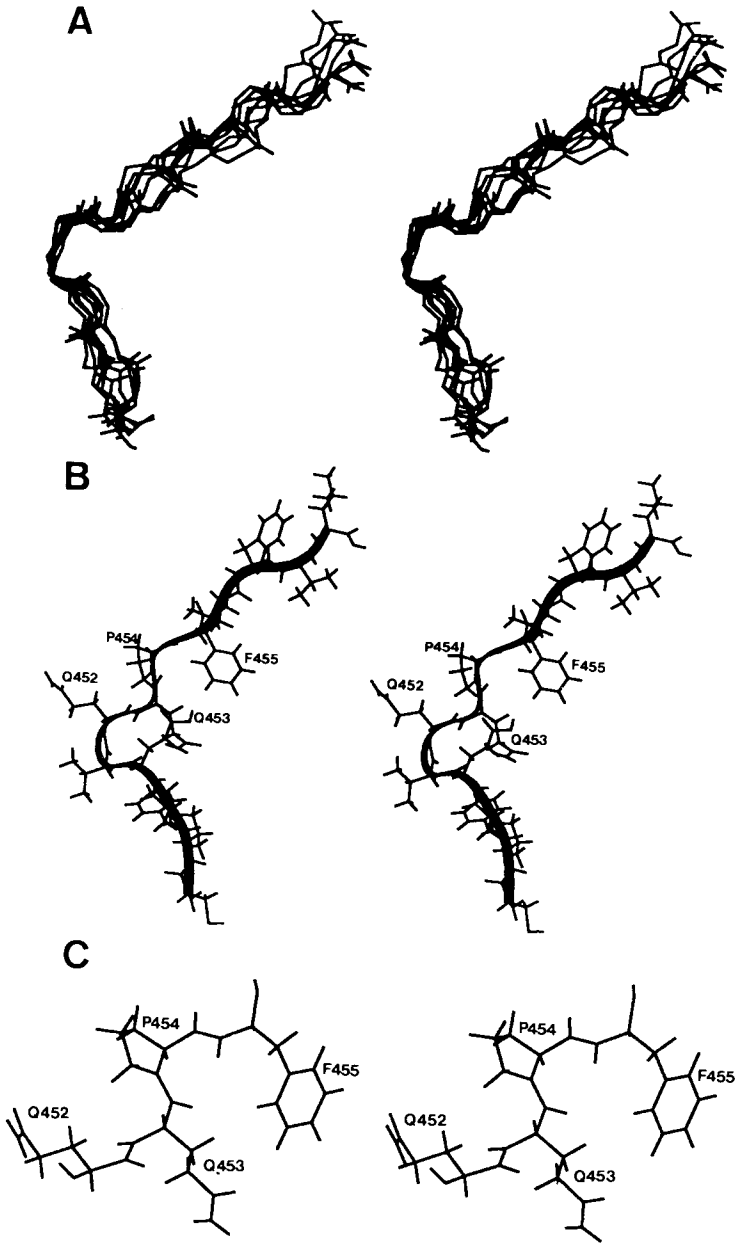


Fig. 3. NMR solution structures of peptide 2. The NMR sample of the peptide was dissolved in water of 50 mM sodium phosphate containing 10% (v/v) DMSO, with pH 7.6 to a final concentration of 14 mM. All NMR spectra were recorded on a Varian Unity 500 MHz NMR spectrometer at 25C. The complete resonance assignments of the NMR spectra were achieved by following the connectivity along the entire peptide with the combination of TOCSY and NOESY spectra [17–22]. Molecular modelling was carried out by using the simulated dynamics [24–27] of the Biosym DISCOVER program with the distance constraints from NOESY spectra [28–30]. A: Superimposition of eight conformations of the peptide. B: Plots of the structure with side chains of Gln^{452}–Gln^{453}–Pro^{454}–Phe^{455} displayed in the rigid region of the peptide. C: More detailed structure of the conformation of the region comprising Gln^{452}–Gln^{453}–Pro^{454}–Phe^{455} residues.

F455 (Fig. 3), with the ends of the peptide exhibiting flexible folding tendencies. The side chains of Q452, Q453 and F455 are all exposed to the solvent, in agreement with our computer model of the intact serpin domain of C1-inh (Fig. 4).

The replacement of either Q452 or Q453 by glutamate greatly reduced C1s binding, indicating that both residues serve as hydrogen bond donors rather than acceptors. Thus it is possible that the binding of these residues to C1s is associated with a cluster of negatively changed residues in C1s. The interaction of these C1s residues with the secondary binding site on C1-inh would help to maintain a tight hydrophobic contact between enzyme and inhibitor in the primary interaction site. Although the mechanism by which the SDS-resistant serpin–enzyme complex is formed is not yet fully understood, the maintenance of such a hydrophobic contact between an enzyme and a serpin is probably essential for the inhibitory function of the serpins, as it excludes small molecules (e.g. water molecules) from the acyl group formed between inhibitor and enzyme.

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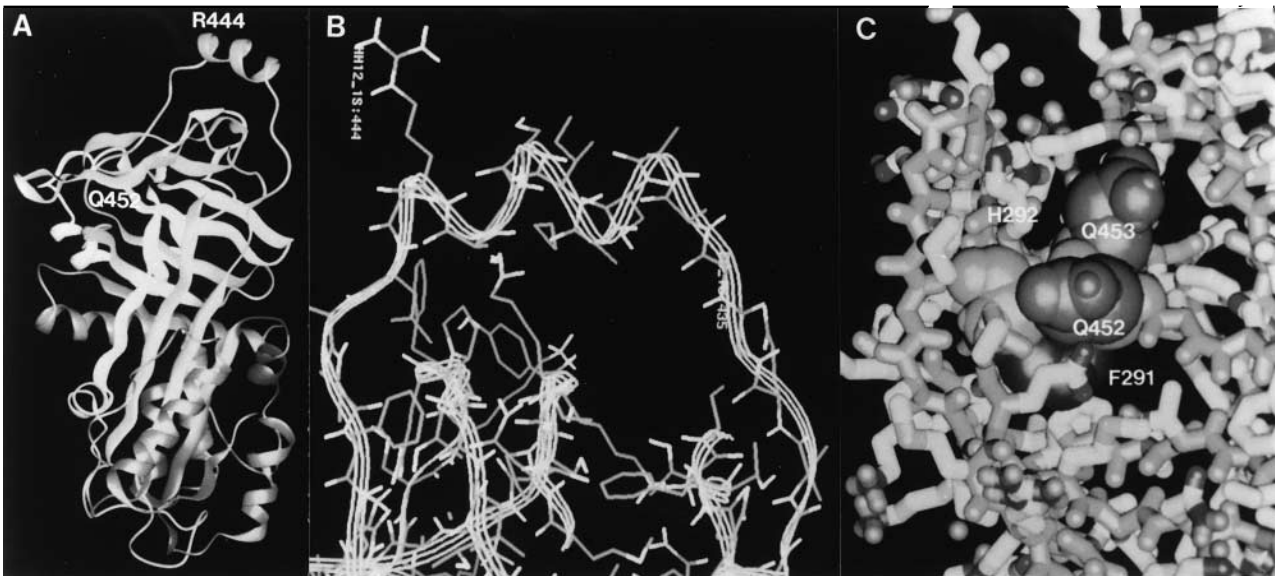

Fig. 4. Computer modelling of C1-inh molecule. A C1-inh model was created with Silicon Graphics using ovalbumin [23] coordinates. When differences in amino acid sequence occurred, the ovalbumin residue was replaced by the C1-inh residue [12]. The results were confirmed using antithrombin III data [24]. A: Serpin domain model of C1-inh based on ovalbumin crystal structure. The reactive site residue (Arg^{444}) is labelled. The proximal hinge is to the right of the reactive site residue and the distal hinge is to the left. One of the residues of secondary C1s binding site (Gln^{452}) is labelled. B: Close-up view of reactive site loop of C1-inh showing side chains of amino acid residues, with Arg^{444} labelled. C: Close-up view of the secondary C1s binding site showing aromatic ring Phe^{291} and the indole ring of His^{292}. The nitrogen-containing residues of Gln^{452} and Gln^{453} are shown. The aromatic ring of Phe^{455} is located to the right of Gln^{453} (unlabelled).

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