# Combined High-Resolution Neutron and X-Ray Analysis of Inhibited Elastase Confirms the Active-Site Oxyanion Hole but Rules Out a Low-Barrier Hydrogen Bond

To help resolve long-standing questions regarding the catalytic activity of the serine proteases, the structure of porcine pancreatic elastase with its inhibitor has been analyzed by high-resolution neutron and X-ray crystallography. The 1.65 Å resolution neutron and 0.94 Å resolution X-ray data show that the hydrogen bond between His57 and Asp102 is 2.60 Å in length and that the hydrogen-bonding hydrogen is 0.80–0.96 Å from the histidine nitrogen. This is not consistent with a low-barrier hydrogen which is predicted to have the hydrogen midway between the donor and acceptor atom. Neutron analysis also shows that the oxygen of the oxopropyl group of the inhibitor is present as an oxygen anion rather than as a hydroxyl group, supporting the role of the "oxyanion hole" in stabilizing the tetrahedral intermediate in catalysis.

Almost one-third of all proteases can be classified as serine proteases, named for the nucleophilic serine residue at the active site. Three amino acids, histidine, aspartic acids, and serine (His57, Asp102, and Ser195; residues are numbered based on homology to chymotrypsin), compose the catalytic triad conserved in the active site of serine proteases. The nucleophilicity of Ser195 is hypothesized to increase via a "low-barrier hydrogen bond" (LBHB) formed between the side chains of His57 and Asp102. The tetrahedral intermediate is electrostatically stabilized through hydrogen bonds with the backbone amides of Gly193 and Ser195, together forming an "oxyanion hole". However, the LBHB hypothesis in serine protease catalysis is still a matter of debate, and the state of the oxygen atoms of the substrate in oxyanion holes has not been confirmed yet. To help resolve long-standing questions regarding the catalytic activity of the serine proteases, the structure of porcine pancreatic elastase (PPE) has been analyzed by high-resolution neutron and X-ray crystallography [1].

For neutron diffraction study, a crystal with dimensions of 2.9 × 1.4 × 1.2 mm (~3.3 $mm^3$) was obtained by repeated macroseeding under deuterated solution over a period of four months [2]. Neutron data collection under room temperature was performed at the 1G-A site of the JRR-3 reactor, JAEA. The full data set was integrated and scaled to 1.65 Å resolution. The 1.20 Å X-ray data under room temperature using the same crystal after neutron data collection was collected at BL-6A. The tertiary structure of PPE complex with the peptidic inhibitor FR130180, including a total of 4,226 atoms (hydrogen [H] and deuterium [D] atoms: 2,172, non-H and D atoms: 2,054) was determined to 1.65 Å resolution by neutron and 1.20 Å resolution by X-ray crystallography at room temperature in a joint refinement using the program PHENIX from the same crystal (Fig. 1). In addition, the 0.94 Å resolution X-ray structure at 100 K was also determined using another crystal at BL41XU, SPring-8, in order to compare the results.

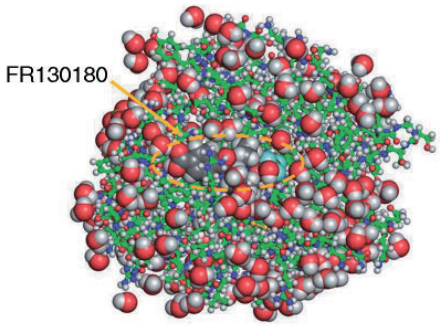


Figure 1
Overall structure of the complex between PPE and peptidic inhibitor FR130180. FR130180, ions and water molecules are shown by space-filling representation. Hydrogen and deuterium atoms are colored in white.





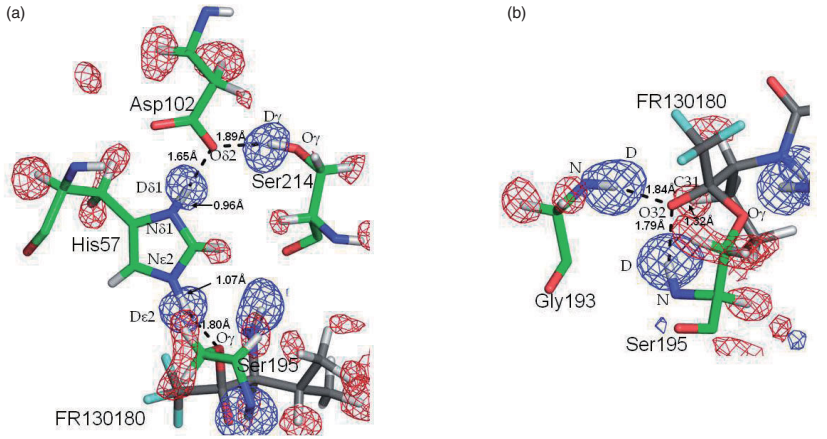


Figure 2
Nuclear density map of the PPE/FR130180 complex at 1.65 Å resolution. (a) Catalytic triad. (b) Oxyanion hole. The *Fo-Fc* nuclear maps were calculated without hydrogens and deuteriums. The blue and red density contours show +5.0 σ and –4.5 σ densities, respectively. Hydrogen (red) and deuterium (blue) were identified according to each atomic scattering length by neutrons.

The FR130180 is covalently bound to the side chain of Ser195 of PPE. In this covalent complex, the carbonyl structure in FR130180 is converted to the tetrahedral structure mimicking the catalytic transition intermediate state. The 2.60 Å distance between Nδ1 of His57 and Oδ2 of Asp102 in catalytic triad in both neutron and sub- angstrom X-ray structures is suggested to be a LBHB according to the previous definition. However, the hydrogen bonding hydrogen between His57 and Asp102, which was confirmed on the $F_o$–$F_c$ nuclear [Fig. 2(a)] and electron density maps, is 0.96 and 0.80Å from the histidine nitrogen, respectively. This is not consistent with a LBHB which is predicted to have the hydrogen midway between the donor and acceptor atom. The observed interaction between His57 and Asp102 is essentially a short but conventional hydrogen bond, sometimes described as a short ionic hydrogen bond.

The carbonyl oxygen (O32) of the oxopropyl group of FR130180 is converted to a hydroxyl oxygen that interacts with the oxyanion hole comprising two hydrogen atoms from the backbone amides of Gly193 and Ser195. These two hydrogen atoms (deuterium in the neutron structure) are clearly confirmed in the $F_o$–$F_c$ nuclear density maps [Fig. 2(b)]. The nuclear density maps also show that the oxygen (O32) is present as an oxygen anion rather than as a hydroxyl group, supporting the role of the oxyanion hole in stabilizing the tetrahedral intermediate in catalysis. The distance between C31 and O32 is 1.32 Å in the sub-angstrom X-ray structure by non-restrained refinement. This distance corresponds to the medium value between typical distances of C=O (1.21 Å) and C–OH (1.43 Å) bonds.

This is the first time that an oxyanion of an intermediate analogue has been directly observed at an oxyanion hole. The precise structural information including hydrogen positions will help clarify the catalytic mechanism of serine protease, and the structural information including hydrogen positions will be useful for designing inhibitors for preventing the fatal disease pancreatitis.

## REFERENCES

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## BEAMLINE

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