Angiography contrast agents interact with serine proteinases The molecular structure of the model system elastase/iohexol

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Abstract Pancreatic elastase was co-crystallised with iohexol, a tri-iodo benzenic contrast agent used in angiography analyses. The X-ray analysis of the complex reveals the presence of three molecules of iohexol associated with the proteinase with low occupancy factors. Two iohexol molecules are located in and near the active site of the enzyme and provide a model for explaining the inhibition of the hemostatic system, one of the major and inconvenient side effect associated with these chemicals.

Key words: Iohexol (tri iodobenzoic); Elastase; X-ray Diffraction; Angiography; Proteinase; Inhibition

1. Introduction

Contrast media used for the visualisation [1] of the vascular system (angiography) are neutral or ionic poly-iodo aromatic molecules (Fig. 1) that are injected in large quantities in the blood vessels. They are frequently associated with clinical secondary symptoms relevant to their bioincompatibility: allergic reactions and disturbance of the hemostatic system are the most common side reactions affecting the diagnostics and yet no specific therapeutic prevention can be made on a simple basis.

Several types of contrast media have been developed during the past fifty years: most of them derive from tri-iodo benzoic acid with either the carboxylic group free (ionic sodium salts) or substituted (neutral compounds). Iopamidol and Iohexol are two members of the second group, the so called non-ionic low osmolality family (Fig. 1) and they all are very soluble in water at physiological pH. Quantities as large as 25–30 g are injected for a single analysis. Owing to a high pharmacokinetic rate of elimination, they are excreted within 20–30 min. These doses can be reduced, but only slightly, when operating at the iodine K edge ($\lambda = 0.31$ Å) which enhances the imaging contrast. These chemicals cannot be considered as true biomaterials as they are intended to interact as little as possible with the biological system or organism. However their side reactions on the hemostatic system by inhibiting the clot cascade, clearly indicate that interactions occur between the proteins involved in the reaction and the contrast agent.

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The role played by serine proteinases (e.g. thrombin) in the clot cascade is crucial and several hypotheses were addressed about possible implication of contrast media at this level [2]. The most evident is to consider that inhibition may occur during the hydrolytic reaction of thrombin or associated enzymes following a mechanism related to the well known inhibition reaction of serine proteinases. Recently, the binding of Tri-iodo benzoic acid (TIB) to human serum albumin has been reported [3]. Despite a strong ionic character, TIB is observed in hydrophobic crevices delimited by the folding of two helices. TIB itself is not used as a contrast medium in angiography but many of its derivatives, Ioxithalamate, ioxaglic salts etc. are routinely used. Consequently, equivalent biochemical interactions would be possible during angiography on serum albumin thus explaining some of the unpredictable side effects observed so far. Clearly the contrast media used at high concentration are molecules displaying ubiquitous properties and may well be implicated in many other undesirable reactions, especially in one of the most frequently observed, the clot cascade inhibition.

In order to test the validity of a serine proteinase inhibition, we have investigated the three-dimensional X-ray structure of a model serine proteinase, the Pancreatic Porcine Elastase (PPE) , in the presence of high concentrations ($>10^{-1}$ M) of iohexol, a widely used neutral contrast agent.

2. Materials and methods

All attempts to co-crystallise PPE with iohexol at concentrations above $5 \cdot 10^{-2}$ M failed. The methodology developed was to prepare crystals following the classic batch technique [4] at pH = 5.5 and to let the crystalline iohexol diffuse in the solution. As the dissolution is slow, osmotic shocks are limited. The contrast agent was allowed to react with the protein crystals over a period of several days. Different soaking concentrations were investigated, among them the value of 0.25 M appeared to be an optimum. Above this value the diffraction pattern of the crystals becomes affected in terms of resolution and below a concentration of 0.1 M no modifications are observed, on the basis of the R_{merge} factor calculated at the end of the data recordings. Complete destruction of the crystals occurs above 0.5 M within a two-hour soaking time.

The diffraction data were recorded at a wavelength of 0.9 Å at the LURE synchrotron facility in Orsay, France. Data recordings were made on the wiggler W32 beam light output using an Imaging Plate MAR-Research system. Crystal-to-film distance was set to 160mm. Each frame was one degree rotation with an exposure time = 30s per frame. A total rotation of $\omega = 101°$ was performed. The Iohexol/PPE crystals are highly isomorphous with the native, orthorhombic space group, $P2_12_12_1$ with a = 75.1; b = 57.9; c = 51.3 Å (Z = 4). The intensities were indexed and integrated using the MOSFLM program [5] interfaced with the CCP4 suite of programs [6].

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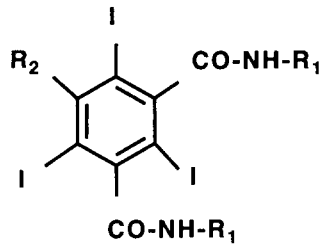

Fig. 1. Iohexol: R_1 = -CH_2-CHOH-CH_2OH; R_2 = -N(Ac)-R_1. Iopamidol: R_1 = -$CH(CH_2OH)_2$; R_2 = -NH-CO-CH(OH)-CH_3.

The localisation of iohexol molecules was made by difference-Fourier calculations using the experimental $|F_{iox}|$ moduli and the ϕ_{nat} phases, derived from the native co-ordinates (ref. 6EST from the Protein Data Bank [7]).

Three residual regions of electron density were found (Fig. 3A,B) showing the characteristic signature of iodine atoms arranged in equilateral triangles. The distances between the different peaks correspond to the iodine-iodine distance previously determined in the high resolution X-ray structure of Iopamidol [8]. Each region of residual density was assigned to a iohexol molecule.

The structure was refined using the XPLOR program [9] and completed by a few cycles of PROLSQ refinements [10]. The iohexol molecules were modelled using a tri-iodobenzoic acid moiety, without attempting to build the unobserved side chains. The temperature factors of the three ligands were fixed to the mean B value of the refined 118 water molecules localised on successive $2F_o - F_c$ maps ($\langle B \rangle$ = 32 Å^2) while their occupancy factors were allowed to refine freely.

Table 1
Refinement parameters for the elastase/iohexol structure

Type of constraints	rms (number of constraints)
Bond distances (1–2) Å	0.018 (1897)
Angle distances (1–3) Å	0.037 (2568)
Planar distances (1–4) Å	0.07 (680)
Planes (Å)	0.007 (325)
Chiral volumes (Å^3)	0.05 (289)
Single contact torsions (Å)	0.23 (421)
Multiple contact torsions	0.29 (870)
Hydrogen bonds (Å)	0.18 (182)
Planar angles (ω etc.)	3.2° (251)
Staggered (±60, 180°)	18° (287)
Orthonormal (±90°)	21° (27)
Average B factors (Å^2)	
Main-chain	18.1 (961)
Side-chains	25.7 (861)
Water molecules	32.1 (125)
R factor	0.192
weights in R factor	83.-122* (s-1/6)
rms shifts in last cycle	
coordinates	0.016 (9)
B factors	1.6 (2) Å^2
No. of fobs used	12,124
Resolution range	18-1.8 Å

The final co-ordinates have been deposited with the Protein Data Bank [7] and are also available by E-mail from the authors, at internet address: prange@lure.u-psud.fr

3. Results and discussion

The three binding sites (Fig. 2) are situated in or near the active site of elastase (Table 2), one is close to the catalytic triad, (subsite S1 – Fig. 4), the second is in the vicinity of Ser^{214} in subsites S2/S3 while the third is located at the surface of the protein in a pocket delimited by the crystal packing and showing important stacking/staggering interactions with the so-called 'aromatic cluster' region of elastase (Tyr^{93}, Trp^{94}, Tyr^{101} and Phe^{215}). This subsite is probably less important, enzymatically speaking, than the other two.

All the three iohexol molecules have low occupancy factors and showed important disorder preventing the localisation of the side chains. The refinement of the structure did not permit their positions to be defined and the omit maps using the calculated phases were essentially void of additional electron density.

Our results indicate that the affinity of iohexol is directed towards the hydrophobic regions of elastase. Of particular interest is the first ligand (noted Iox-1) located in the active site (Fig. 3A and Fig. 4). The orientation of the catalytic triad is not affected by this bulky molecule and the hydrogen bond usually observed between Ser^{195} and His^{57} in the free enzyme is still present (3.02 Å). The least-squares superimposition of the free and complexed enzymes was performed using all the protein atoms within a sphere 10 Å in radius centered on the active Ser^{195} (415 atoms – program XPLOR). The root mean square deviation does not exceed 0.44 Å. As a consequence, the iohexol

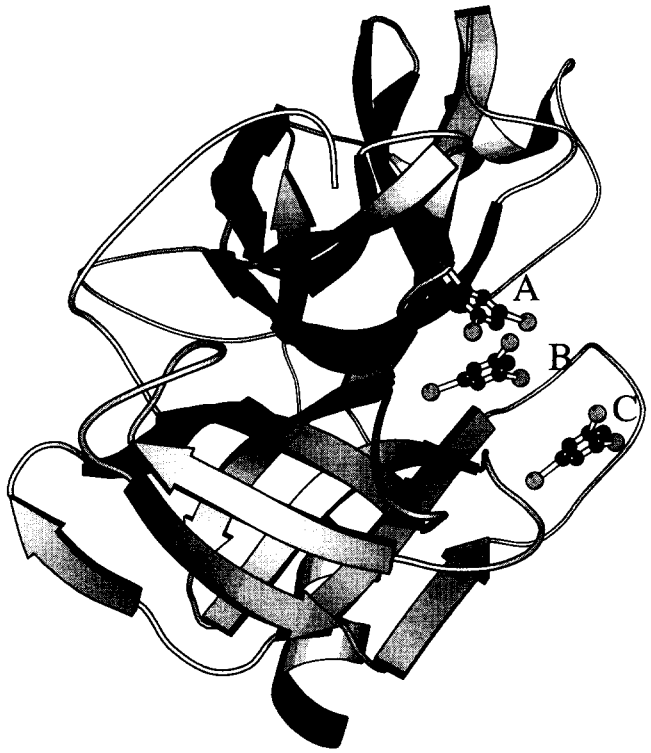

Fig. 2. Overall view of elastase structure showing the two domains (N-terminal, lower and C-terminal upper) and the locations of the three iohexol molecules noted A, B and C. Only the tri-iodobenzene rings are represented. (Richardson's representation using the MOLSCRIPT program [12]).

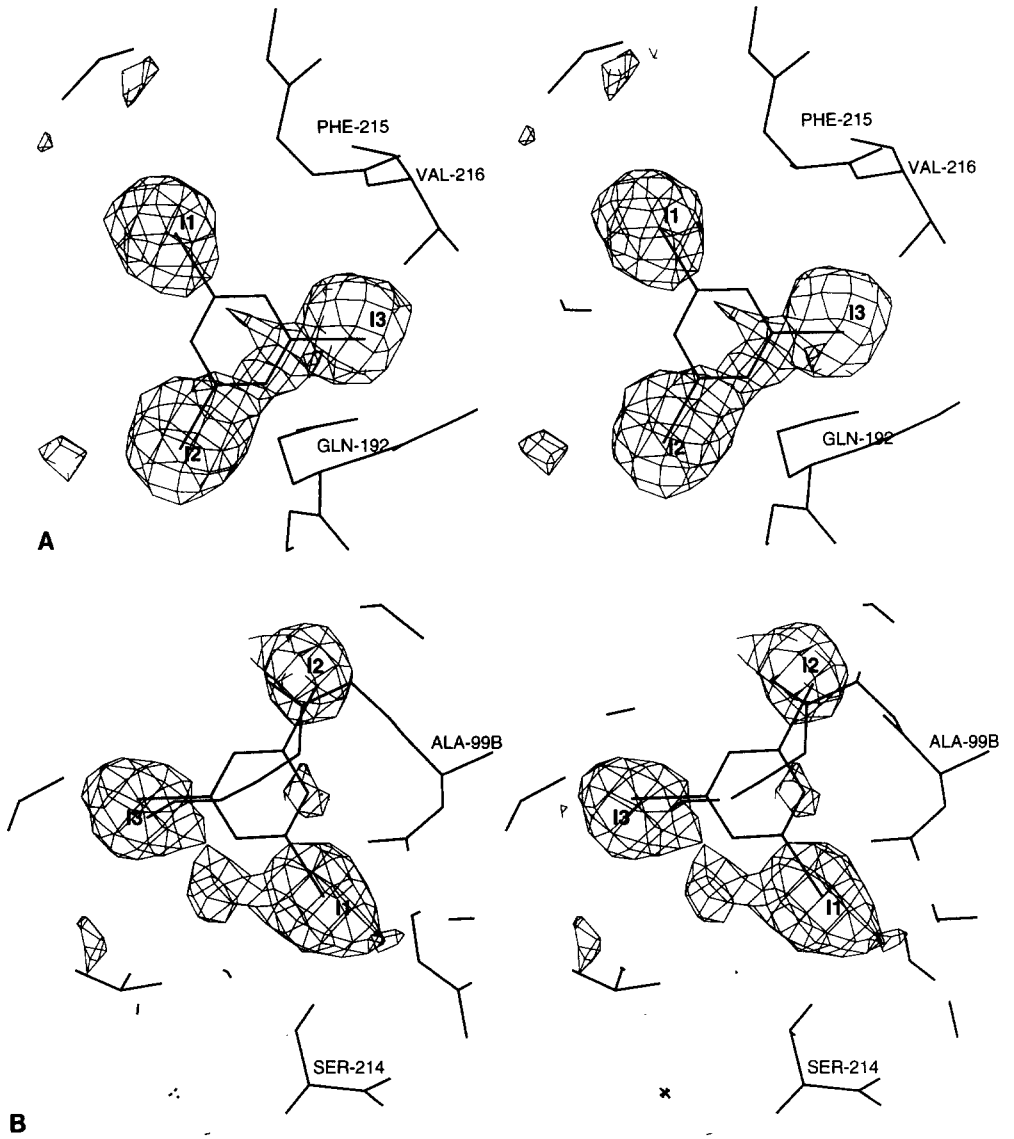

Fig. 3. Stereo view of the difference-Fourier electron densities corresponding to the iohexol molecules in subsites 1 and 2 , superimposed with the tri-iodobenzene model. The contouring is at 2σ of the average density.

molecule located in the active site just appears to block the catalytic pocket. This effect is probably at the origin of the significant decrease of the hydrolysis rate observed in solution by kinetic studies.

Table 2
Aminoacids in the vicinity (2.8–5 Å) of the three iohexol molecules

Iox-1			Iox-2		Iox-3	
Thr^{41}	Asp^{102}*	Thr^{213}	Val^{99}	Thr^{175}	Tyr^{93}	Lys^{177}
Cys^{42}		Ser^{214}	$Ala^{99}A$	Lys^{177}	Trp^{94}	
	Gly^{190}	Phe^{215}	$Ala^{99}B$		Asn^{95}	Asp^{98}
His^{57}*	Cys^{191}	Val^{216}	Gly^{100}	Ser^{179}		Val^{99}
	Gln^{192}		Tyr^{101}	Met^{180}	Arg^{36} #	$Ala^{99}A$
Val^{99}	Gly^{193}		Asp^{102}*			$Ala^{99}B$
	Asp^{194}			Ser^{214}		Gly^{100}
	Ser^{195}*		Thr^{229}	Phe^{215}		
			Arg^{230}			
				Tyr^{234}		

The refined occupancy factors are 31%, 23% and 39%, respectively. Each molecule was assigned a temperature factor equal to 32 Å^2. *Catalytic triad; #from another symmetry-related molecule.

Hydrolysis rate of succinyl-Ala_3-p.NO_2-anilide as substrate by elastase, has been determined in presence of several contrast agents: iohexol, iopamidol, ioversol and iopromide [11]. At a concentraton of 100mg Iodine/ml (corresponding to 0.26 M) the K_m increases by a factor of two while K_{cat} remains unchanged, indicating a competitive inhibition of elastase.

The second ligand (noted B in Fig. 2) – Fig. 3B – is located in the S2/S3 subsites of the active site crevice. It interacts with the two hydrophobic loops 94–103 and 212–216 both delimiting the active site. Its presence though disordered and its interaction probably non specific, may reinforce the kinetic influence of the first molecule located in the active site.

This inhibition of elastase, used as a model enzyme, gives additional support to the hypothesis of a potent inhibition of thrombin, one of the most dramatic side effects observed during angiography analyses.

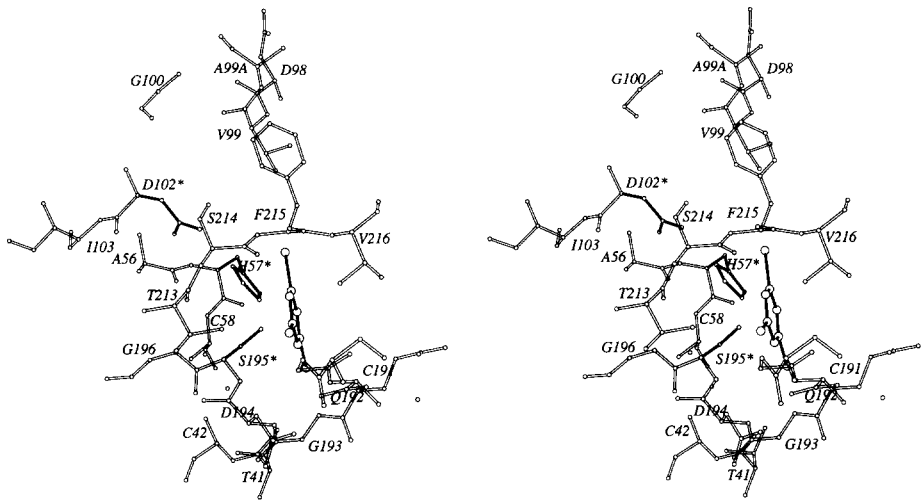

Fig. 4. Subsite 1: the position of the aromatic tri-iodo ring of iohexol in the active site of PPE (the lateral chains of iohexol are not drawn). The three amino acids of the catalytic triad, Ser^{195}, His^{57} and Asp^{102} are situated at left in bold tracing.

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