Study of the Complex Between the Contrast Agent Iobitridol (Xenetix®) and Elastase (PPE): A Model for Hydrophobic Site Protection in Drug–Protein Interactions

Thierry Prangé,[1,3] Alain Neuman,[1] Claire Corot,[2] and Dominique Meyer[2]

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Purpose. The concept of Hydrophilic Sphere Stabilization, or Hydrophobic Shielding, has been postulated in the synthesis of biocompatible contrast agents in vascular imaging. To improve the safety of these polyiodinated agents, interactions with protein hydrophobic sites in biomacromolecules should be kept as low as possible. In order to evaluate the level of interactions with proteins, we have selected the serine proteinase Elastase, in presence of Iobitridol (Xenetix®), as a model.

Methods. The complex between Iobitridol and Pancreatic Porcine Elastase was investigated by X-ray diffraction techniques, on saturated monocrystals, using the synchrotron radiation at 0.98Å.

Results. In contrast to Iohexol, which displays several interactions including one in the active site, Iobitridol is unable to interact directly with elastase. Only one partially occupied site is found in between two molecules of the crystal packing.

Conclusions. The validation of the "hydrophobic shielding" concept, which was at the origin of the design of the Iobitridol molecule, has been proven to be an essential feature in minimizing *in vivo* protein interactions.

KEY WORDS: iobitridol; proteinase; hydrophobic shielding; contrast agent; X-ray diffraction.

INTRODUCTION

In contrast to therapeutic agents, research on contrast agents for medical imaging aims at minimising their capacity to interact with biological sites and bind to them. Indeed, the interactions of iodinated contrast agents with proteins and membranes is believed to be one of the causes of their unwanted effects (for a review see (1)).

Because the interaction of non-ionic contrast agents (LOCM, low osmolar contrast media) with biological sites is essentially of a hydrophobic nature, its intensity is related to the accessibility of their lipophilic zones, in particular the 1,3,5-triiodobenzene ring (TIB). This has led to a variety of strategies to mask such lipophilic zones mainly by introducing hydrophilic side chains. Biological interactions of a non-ionic LOCM will be minimal if the hydrophilic contribution of the polar chains is distributed symmetrically around the triiodobenzene ring to create, in effect, a hydrophilic sphere. Furthermore, the conformational behaviour has to be taken into account since a dynamic stabilisation of the hydrophilic sphere is necessary to prevent hydrophobic forces from unmasking the TIB ring (1). Based on these considerations, a novel non-ionic LOCM, iobitridol, has been designed (Patent EP 437144).

Since it has been demonstrated that non-ionic LOCM may interfere with the serine proteinases of the clotting cascade (2), PPE (Porcine Pancreatic Elastase) which belongs to the same family has been used as a model for studying the hydrophobic interactions of these compounds. This protein can be crystallised and its 3D structure is known at a resolution of 1.7Å (3). By X-ray diffraction on single crystals soaked in high concentration solutions of contrast agents, it is possible to visualise the sites of interaction at the molecular level.

In this present work, we describe the interaction site of Iobitridol and compare this result with the interactions already observed in the case of the less hindered Iohexol molecule (4).

MATERIAL & METHODS

Elastase (PPE) was purchased from SERVA Chemicals or Fluka company as a lyophilised powder. The native crystals were obtained using the described procedure (5) by dissolving 1.4 mg of elastase in 100µl of acetate buffer (pH = 5.5) and adding 1 µl of sodium sulphate as precipitant agent. Crystals usually appear within a few days and grow up to 0.7 mm in dimension. Most of the crystals are elongated prismatic rods of 0.2×0.2×0.5mm in size.

Saturation by Iobitridol was achieved following the soaking procedure: the quantity of Iobitridol corresponding to a final 0.5M solution was added directly as a solid powder to the crystallisation mother liquor in equilibration with one selected crystal. The slow dissolution of the powder minimise the osmotic shock on the crystal. After a soaking time of two weeks at 4°C, the crystal was harvested out of the solution and mounted in a glass thin-wall capillary with a drop of mother liquor at one end to avoid dehydration of the crystal. Crystals are orthorhombic, space group: $P2_12_12_1$, cell parameters: a = 51.33, b = 57.50 and c = 74.87 Å.

The X-ray diffraction patterns of the Iobitridol soaked crystal were recorded on an MAResearch Image Plate system at the W32 beam line station of the DCI synchrotron facility in Orsay, France. The diffraction data consist of 100 frames of 2 degree rotation each. The diffraction spots were indexed and integrated using the MOSFLM program (6) and the resulting intensities were merged and scaled with the CCP4 package of programs (7). Table 1 summarises the processing of the diffraction amplitudes.

The Iobitridol site(s) were investigated by difference-Fourier calculations using the native structure as reference (known native structures were from the Protein Data Bank (8): code for Elastase: 6EST) and using the $|F_{deriv}|-|F_{native}|$ terms as amplitudes and ϕ_{native} as phases. Only one well defined site was observed (Figure 2) in the difference map.

Structure refinements of PPE/Iobitridol were carried out using the stereochemically restrained least-squares minimisation method used in the PROLSQ program (9). The dictionary

[1] Chimie Structurale Biomoleculaire (URA-1430 CNRS) F-93017 Bobigny Cedex, France.

[2] GCA Service Immuno-Hemato-Biochimie, Z.A. De l'Islon F-38670 Châsse sur Rhône, France.

[3] To whom correspondence should be addressed. (e-mail: prange@lure.u-psud.fr)

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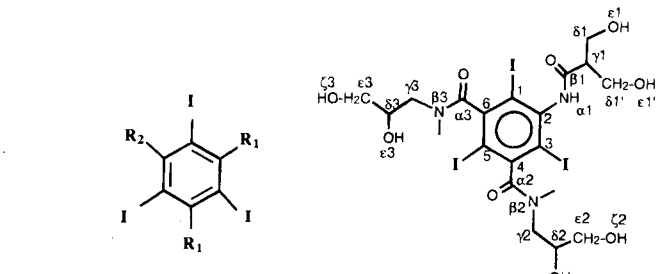

Iopamidol : R_1= -CO-NH-CH(CH_2OH)$_2$
R_2= - NH-CO-CH(OH)-CH_3

Iohexol : R_1= -CO-NH-CH_2-CHOH-CH_2OH
R_2= - N(Ac)-R

Iobitridol : R_1= - CO-N(CH_3)-CH_2-CH(OH)-CH_2OH
R_2= -NH-CO-CH(CH_2OH)$_2$

Fig. 1. Chemical structures of Iopamidol. Iohexol (Omnipaque®) and Iobitridol (Xenetix®). The three molecules belong to the non-ionic, low osmolality, family and share the same central triiodobenzene ring, hindered by three side chains of different lengths. Hydroxyl functions are important to achieve a high solubility (in molar ratio) in physiological media.

of chemical constraints for the Iobitridol fragment was prepared according to the published high resolution structure of some close parents (10). However, the precise locations of the Iobitridol side chains are not possible for at least two reasons: the molecule displays, at room temperature, several different stabilised conformational states due to high rotational barriers (11) and furthermore Iobitridol is present as a racemic mixture of four diastereoisomers. However most of the atoms can be modeled in the electron density, with the exception of four atoms: two N-methyl groups and one hydroxymethyl function (ϵl′).

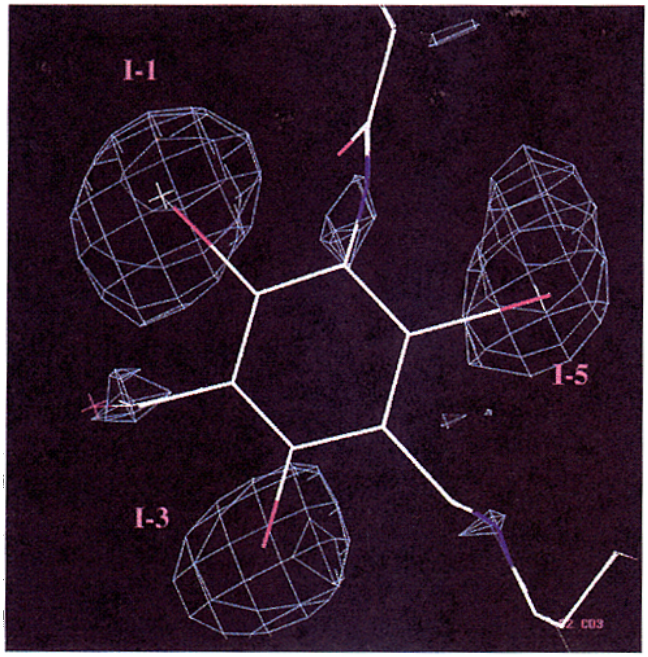

Fig. 2. Initial difference-Fourier electron density calculated with amplitudes = $|F_{deriv}-F_{native}|$ and phases = ϕ_{native}. The contouring is at the 2sigma level above the mean. The molecular model of the Iobitridol molecule is shown superimposed.

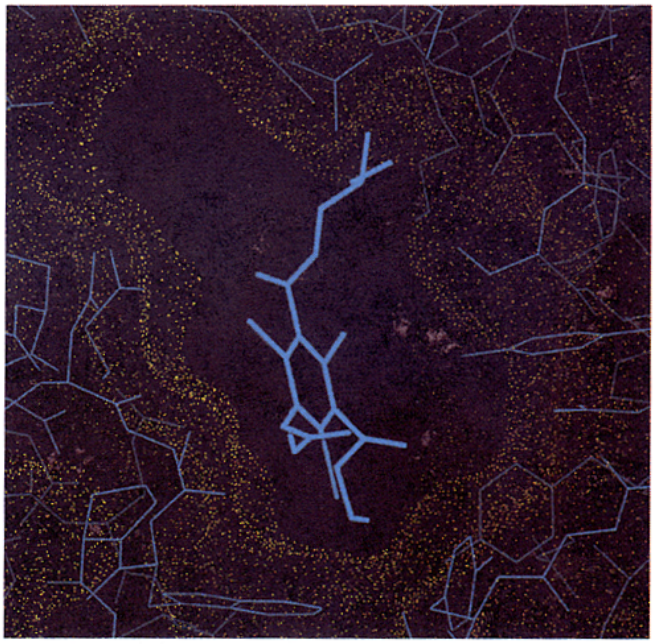

Fig. 3. The intermolecular pocket where the Iobitridol molecule is trapped. The surface is calculated according to (12). The molecule occupies one part of the large cavity built in the crystal packing, the other part being filled by the sulphate ion and water molecules.

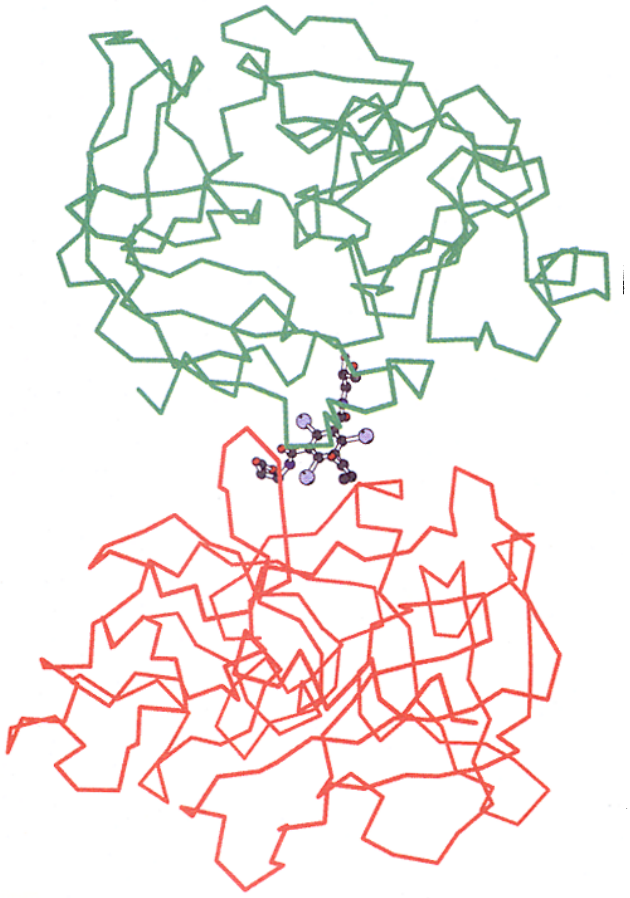

Fig. 4. The position of the Iobitridol in the packing between two symmetry related molecules of elastase (represented in Cα tracing).

Table 1. Data Processing and Refinement of the X-ray Diffraction of Elastase/Iobitridol Soaked Crystals

Wavelength:	0.98 Å
$\Delta\omega$ Rotation per frame:	1°5
Exposure time/frame:	45s
Overall ω angular domain:	110°
Crystal-detector Distance:	270 mm
nominal resolution:	1.8Å
Total nb. of recorded intensities:	52128
Nb. of independent intensities:	15405
R_{symm}on symmetry related intensities:[a]	0.068
Practical resolution:	1.9Å

[a] defined as $R_{symm} = \frac{\Sigma\Sigma|Fi-\langle Fi\rangle|}{\Sigma|\langle Fj\rangle|}$.

Table 2. Refinement Parameters for the Elastase/Iobitridol Structure

Type	nb.	r.m.s.
Nb. of atoms	2006[a]	
Distances 1–2	1950	0.020 Å
Valence angles (dist. 1–3)	2559	0.038 Å
Diehedral angles (dist. 1–4)	680	0.053 Å
Planes	323	0.018 Å
Chiral volumes	289	0.18 Å^3
simple/multiple torsion contacts	1132	0.25 Å
Plane angles (0/180°)	281	3.5°
Staggered angles (±60/180°)	287	16°
Orthogonal angles (±90°)	27	18°
Hydrogen bonds	159	0.18 Å
average ⟨B⟩ (main chain)	19.1 Å^2	(min/max:3./44.)
average ⟨B⟩ (side chains))	25.6 Å^2	(min/max:3./49.)
B factor of Iobitridol	27 Å^2	
Occupancy factor of Iobitridol	0.58	
Resolution limits	5-1.9 Å	
nb. of used Fobs ($\geq 4\sigma$)	14.428	
Final R (on Fobs)/R_{free} (%)	17.6/24.1[b]	

[a] including: 1822 protein atoms, 1 calcium ion, 1 sulfate ion, 30 atoms for Iobitridol and 148 water molecules.

[b] calculated as $R = \frac{\Sigma|\,|F_{obs}| - |F_{calc}|\,|}{\Sigma|F_{obs}|}$.

The native structure inclusive of the previously located Iobitridol molecule and exclusive of water molecules was used as the initial model. At regular intervals, water molecules were identified by difference-Fourier calculations and their contributions added to the model. The final statistics and R-factors calculated at the end of the refinements are also included in Table 1. The final coordinates have been deposited with the Protein Data Bank and are also available by e-mail from the authors at: prange@lure.u-psud.fr.

RESULTS AND DISCUSSION

The difference-Fourier electron density (Figure-2) clearly reveals the triangular signature of the three iodine atoms of the TIB core of Iobitridol. However the density is relevant only to a partially occupied site. As atomic thermal factors are strongly correlated with the occupancy factors, it was decided during the refinement to fix the B factors to the average <B> terms deduced from the water molecules of the first shell (i.e. directly bonded to the elastase molecule), and to let an overall occupancy factor refine freely for all of the atoms of the Iobitridol molecule. A final value of 0.58 was obtained. Further difference-Fourier calculations did not reveal additional sites of Iobitridol. The occupancy factor is high enough to permit part of the side chains of Iobitridol to become visible, contrary to what happened in the case of iohexol (4). However, important disorders are still evident in the side chains.

The single Iobitridol molecule is found close to the C-terminal helix (residues 234 to 245)[4] and also in the neighbouring of the so-called "aromatic cluster" (built by the side-chains of Y82, Y93, Y101, Y117, Y137, Y159, Y234 and W27). Only a few number of direct contacts are observed, they are reported in Table 3 (within a radius of 3.5Å). The location of the Iobitridol molecule is shown in Figure-3 (cavity) and Figure-4 (general view in between three elastase molecules of the crystal packing).

As mentioned above, only one molecule of Iobitridol is observed in interaction with the elastase molecule. However this interaction cannot be assessed as a classic drug-protein interaction because the site is an intermolecular cavity built by symmetry related molecules in the crystal packing, and it may be just a "crystal trap", characteristic of the crystalline state of the protein.

In the complex structure, among the short contacts, two are hydrogen bonds directed toward water molecules, three are polar contacts directed toward the protein. Only one hydrophobic interaction is observed (Cβ of A126). Among the other contacts, the short distance Cδ1(Iobitridol)-W07 is rather short and probably suspicious because the density of W07(symmetry-related) is not firmly established to be an independent water molecule and may alternatively be attributed to the end of one of the disordered side chains of Iobitridol. In this context, it would become an intramolecular bond. The most important feature is the absence of the TIB stacking or staggering interactions with the "aromatic cluster" as observed in Iohexol complex structure. The figure-4 shows that the Iobitridol molecule is blocked in the crystal packing between two elastase molecules related by the symmetry operators [1 000] and [4 0$\bar{1}\bar{1}$] (or in positional parameters: X, Y, Z and −X, Y−1/2, −Z−1/2).

Table 3. Contacts Between Elastase and Iobitridol

Atoms[a]/residue	distance (Å)	symmetry[b]	Type
Oγ1—W07-258	2.73	**1** 0 0 0	hydration
Oδ1—W07-254	2.76	**4** 0-1-1	?
Oε2—Oζ(Tyr207)	3.13	**4** 0-1-1	H bond
Oζ2—Nε2(Gln157)	3.09	**4** 0-1-1	H bond
Oζ3—Cβ(Ala126)	3.10	**1** 0 0 0	hydrophobic
Oζ3—W03-254	3.12	**1** 0 0 0	hydration
Oζ3—Oγ(Ser236)	3.11	**1** 0 0 0	H bond

[a] The numbering of Iobitridol is given figure-1.

[b] Numbering of symmetries are those of the $P2_12_12_1$ space group: **1**=x,y,z; **2**=1/2+x,1/2−y,−z; **3**= 1/2−x, −y,1/2+ z and **4**= −x, 1/2+y, 1/2−z, followed by the three translation vectors.

[4] The numbering of amino acids follows the standard numbering of chimotrypsinogen (starting at 16 in elastase).

Interactions of contrast media with proteins were investigated intensively and requirements were analysed, based on various analytical approaches (11). The authors evidenced the role of hydrophobic interactions with the TIB ring and underlined the necessity to get them at the lowest possible level. The method they developed was to increase and stabilise a hydrophilic sphere around the TIB core by adding polyhydroxylated carbamoyl chains around, thus preventing hydrophobic forces from the active sites of proteins, or in any other site of interactions.

These interactions usually proceed via cavities or pockets of their target macromolecules. A large number of works has been devoted to cavities in proteins and a short definition may be recalled as following:

A *cavity* is a region in a protein that is not occupied by protein atoms and that is normally entirely closed off by the protein. In the static description of a crystal structure, a cavity is *not accessible* from the outside. In contrast, a *pocket* is connected (*accessible*) from the outside. Most of the catalytic sites in proteins belong to this second type. In order to test whether binding sites are cavities or pockets, molecular surfaces are very often analysed with the Connolly algorithm (12). These cavities or pockets are also classified according to their hydrophilic or hydrophobic character and recent survey on a large number of protein structures has shown that the cavities correspond to about 2% of the total protein volume (13). Thermodynamic considerations also suggest that true hydrophobic cavities do not contain water molecules (14). It is well known that the atoms in the interior of protein molecules are densely packed. Calculations by Klapper (15) have estimated that there is twice as much free volume distributed throughout simple organic liquids than in proteins. However, in proteins the free volume is not distributed randomly (as in liquids) and empty intramolecular cavities exist in numerous proteins (13,16). These cavities exist at the considerable expense of free energy so it is unlikely that they are a consequence of packing defects. The hypothesis that cavities are important for the conformational flexibility of protein molecules is supported by the characteristics of small hydrophobic organic molecule binding to myoglobin or lysozyme, two proteins widely used as examples. Xenon atoms (or krypton in a lesser extend) are useful hydrophobic probes in analysing such interactions (16). In the case of Myoglobin (17), hen egg lysozyme (18) and the phage T4 lysozyme (19), it has been shown both by nmr or X-ray diffraction methods that the inner cavities can accommodate many different ligands like benzene, indole, dichloromethane (20) or chloroform, due to protein core fluctuations on the 10^{-4}s time scale (21). In myoglobin, Tilton *et al.* (22) have observed an overall reduction in temperature factors upon xenon binding, an effect that was interpreted as a ligand-induced restriction of the number of conformational states. Such an interpretation would also explain why the rotational degrees of freedom of bound water molecules in the protein decrease upon ligand binding (17). Binding of xenon or other hydrophobic small ligands to myoglobin or adenylate kynase (23) cavities also affects the functionality of the protein in a rather drastic way (17,24).

In a general study of the anaesthesia mechanisms (25) several authors have focused attention on small organic or gaseous ligands including rare gases (26). Hydrophobic interactions have been postulated to be one of the main mechanisms (27). Several observations support this concept in channel pore structures as represented by Ni/Fe hydrogenase family (28) or the COMP matrix protein (29) closely related with the nicotinic Acetylcholine Receptor structure.

As far as contrast agents are concerned, triiodobenzoic acid (TIBA) is a prototype molecule in the evaluation of the hydrophobic interactions between proteins and the naked triiodobenzene ring. The negative charge of the carboxylate does not seem to play a role, as observed in the case of human serum albumin crystals (30) where such a complex was observed and used in solving the structure. Iopamidol and Iohexol (Figure-1) were two neutral contrast agents, first developed to minimise these hydrophobic interactions. In a previous study (4), we have demonstrated that non-specific interactions with elastase are still present when iohexol is the ligand. However, it was necessary to limit the concentration to a value of 0.25M. At upper concentration, the crystals are no longer stable and destruction occurs within a few minutes. Three different sites of iohexol, with a low (partial) occupancy factor were identified, including one molecule in the catalytic site itself. It was concluded that this site of interaction was responsible for a diminution in the catalytic activity of the enzyme observed by kinetic experiments (31). The active site of serine proteinases is known to have an important hydrophobic character, as shown by its ability to fix xenon (32). Further, the main interaction of iohexol toward elastase (the highest occupied site) is performed through aromatic ring staggering effects with the side chains of several tyrosines of the so-called "aromatic cluster", situated at the protein surface. In this context, it was expected that, protecting the TIB core of the contrast agent with bulky side chains would diminish these interactions. The concept of "hydrophobic shielding" was postulated (11) and is at the origin, the design and the synthesis of Iobitridol (or Xenetix®). In this new molecule, the lengths of branched side chains were increased and amido groups were methylated with the idea to bend the chains out of the TIB plane, over the ring, to protect it *permanently* from hydrophobic interactions.

This is also supported by the higher stability that elastase crystals show when the concentration of Iobitridol is increased to 0.7M (three times the limit observed for iohexol).

CONCLUSION

In the present study, the binding of Iobitridol to pancreatic porcine elastase has been studied by X-ray diffraction. This protein was selected because it represents a model for the contrast agent proteinase interactions. The X-ray structure of elastase crystals equilibrated with a 0.5M concentration of Iobitridol reveals only one, partially occupied, molecule blocked in the crystal packing, with only a few number of contacts directed toward the protein. Compared to iohexol, Iobitridol is much less active and the concentration can be increased by a factor of two before the destruction of the crystals. It is not surprising that Iobitridol has a higher occupancy in its unique intermolecular site, because of the higher concentration used, compared to iohexol. Contrary to Iohexol, no molecule was observed in the active site of the protein, thus giving support to the concept of "hydrophobic shielding", which was at the origin of the design of Iobitridol.

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