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Exploring Hydrophobic Sites in Proteins With Xenon or Krypton

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ABSTRACT **X-ray diffraction is used to study the binding of xenon and krypton to a variety of crystallised proteins: porcine pancreatic elastase; subtilisin Carlsberg from *Bacillus licheniformis;* cutinase from *Fusarium solani;* collagenase from *Hypoderma lineatum;* hen egg lysozyme, the lipoamide dehydrogenase domain from the outer membrane protein P64k from *Neisseria meningitidis;* urate-oxidase from *Aspergillus flavus,* mosquitocidal δ-endotoxin CytB from *Bacillus thuringiensis* and the ligand-binding domain of the human nuclear retinoid-X receptor RXR-α. Under gas pressures ranging from 8 to 20 bar, xenon is able to bind to discrete sites in hydrophobic cavities, ligand and substrate binding pockets, and into the pore of channel-like structures. These xenon complexes can be used to map hydrophobic sites in proteins, or as heavy-atom derivatives in the isomorphous replacement method of structure determination. Proteins 30:61–73, 1998.**

Key words: xenon; krypton; hydrophobic cavity; protein-ligand binding

INTRODUCTION

A large number of proteins are known to fix small exotic ligands, like organic solvent molecules, and several crystal structures of these complexes have been refined and deposited with the Protein Data Bank.[2] The pioneering crystallographic studies of Schoenborn and co-workers demonstrated that sperm whale myoglobin and horse haemoglobin crystals can bind xenon under moderate pressure, through weak van der Waals forces.[55,58,60,75] These xenon complexes were obtained by subjecting native protein crystals to a relatively low gas pressure (2–2.5 bar).§ Xenon was shown to bind to pre-existing atomic-sized cavities in the interior of these globular protein molecules. The interaction of xenon with proteins is the result of non-covalent, weak-energy van der Waals forces,[12,66,67] and therefore, the process of xenon binding is completely reversible.[39,64] The xenon complex with myoglobin has been studied in detail by Tilton and co-workers,[64,66–68] who showed that the number and the occupancies of xenon binding sites vary with the applied pressure. Thus, at a pressure of 7 bar, one major (almost fully occupied) and three secondary (half-occupied) sites were found. Xenon binds to myoglobin with very little perturbation of the surrounding molecular structure. Hence, the xenon complex is highly isomorphous with the native protein structure. Cyclopropane and dichloromethane have also been shown to bind to the major xenon binding site in myoglobin,[42,56,57] but the greater size of these molecules causes some distortions in the surrounding protein structure and rearrangement of some amino acid side chains. Even a single nitrogen molecule binds to this same site at a pressure of 145 bar.[65] Xenon was also described to bind to serum albumin,[9] renin, and tobacco mosaic virus,[57,59] but no structural studies have been undertaken on these complexes.

Due to its anesthetic properties,[11] xenon has been used extensively as a prototype for theoretical and experimental studies on the interactions of anesthetics with proteins.[13,14] The structural investigations of xenon, cyclopropane, and dichloromethane binding to myoglobin and hemoglobin, along with the crystallographic analysis of halothane binding into the enzymatic site of adenylate kinase,[47] provide, so far, good examples of the interaction of general anesthetics to specific sites in proteins. The nature of the molecular site of general anesthesia is still a matter of important debate,[18,38] but over the last decade, evidence has been accumulated in support of the theory that general anesthetics act by binding directly to proteins,[17,18] rather than by perturbing lipid bilayers in synaptic membranes, as it was

§Non SI-units used in this article: 1 bar = 10^5 Pa, 1 Å = 10^{-10} m.

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thought before.[76] More recently, the considerable development in medicine of Magnetic Resonance Imaging (MRI) techniques led to a re-investigation of xenon as a probe in imaging and diagnostic techniques.[30,77]

Protein-xenon complexes can be used as highly isomorphous heavy-atom derivatives for solving the phase problem in X-ray crystallography.[48,50,52,53,63,70] Xenon has now been used successfully as a heavy-atom in the structure determination of the human nuclear retinoid-X receptor RXR-α ligand binding domain,[3] the molybdoenzyme DMSO reductase from *Rhodobacter spheroides*,[54] the oligomerization domain of the cartilage oligomeric matrix protein (COMP),[34] the Photosystem I from *Synechoccus elongatus*,[25] the lipoamide dehydrogenase domain from the outer membrane protein P64k of the bacteria *Neisseria meningitidis*,[33] and the enzyme urate-oxidase from *Aspergillus flavus*.[8]

Until recently, structural studies on protein-xenon complexes had been limited exclusively to the cases of myoglobin and hemoglobin. Binding of xenon into the enzymatic site of serine proteinases has been reported by Schiltz et al.[51] The present study describes and compares the xenon and krypton binding sites in a variety of proteins: porcine pancreatic elastase, subtilisin Carlsberg from *Bacillus licheniformis*, cutinase from *Fusarium solani* (wild type and S120A mutant), collagenase from *Hypoderma lineatum*, hen egg-white lysozyme, the lipoamide dehydrogenase domain of the outer membrane protein P64k from *Neisseria meningitidis*, urate oxidase from *Aspergillus flavus*, mosquitocidal δ-endotoxin CytB from *Bacillus thuringiensis* sp. *kyushuensis* and the ligand-binding domain of the human nuclear retinoid-X receptor RXR-α.† These are proteins whose native three-dimensional structures have been determined by x-ray crystallography and refined to resolutions equal or better than 2.7Å. [3,4,8,26,31,33,35,37,78]

MATERIALS AND METHODS

Sample Preparation and X-Ray Data Collection

Standard procedures, described in the literature were used to grow crystals of elastase,[37] Subtilisin,[44] lysozyme,[62] and P64k.[32] Crystals of cutinase,[35] collagenase,[4] RXR,[3] urate oxidase,[8] and CytB[31] were obtained from the crystallographic groups who solved the structures. For cutinase, the xenon binding was investigated on the wild type protein as well as on the mutant S120A, where the active site serine is replaced by alanine. Crystals of this mutant are isomorphous to the native ones.[36]

The method used to prepare xenon derivatives has been described earlier.[50] Native crystals, mounted in quartz capillaries, fitted to a specially designed cell, were submitted to gas pressure a few minutes before starting the data collection.‡ The gas pressure was maintained during the data collection. Native and derivative data were collected under similar conditions.

Krypton complexes were prepared for elastase and lysozyme at pressures of 48–56 bars.[52]

Diffraction data were collected at the DW32 wiggler beam-line at the LURE synchrotron facility in Orsay, France[16] using x-rays at a wavelength of 0.9 A and a MAR-Research image plate detector. Diffraction data were processed with the MOSFLM program.[29] Data reduction and merging as well as all subsequent crystallographic computations were carried out with programs of the CCP4 package.[6] A summary of the X-ray data collections on all samples is given in Table I.

Xenon and Krypton Site Determination

The coordinates of the major xenon sites were located by difference-Fourier calculations using the $|F_{deriv}| - |F_{native}|$ terms as amplitudes and ϕ_{native} as phases (known native structures were from the Protein Data Bank[2]: codes for elastase, lysozyme, subtilisin, cutinase, collagenase, CytB and RXR were 6EST, 1LSE, 1SCA, 1CUS, 1HYL, 1CBY and 1LBD respectively).

Refinements of the Xenon/Krypton Sites

Structure refinements of elastase/Xe, subtilisin/Xe, collagenase/Xe, urate oxidase/Xe, and lysozyme/Xe complexes were carried out by the method of simulated annealing with the XPLOR program.[5] The native structures inclusive of the previously located xenon/krypton atoms and exclusive of water molecules were used as starting models. At regular intervals, water molecules were identified by difference Fourier-calculations and added to the model. As occupancies and thermal factors are highly correlated, the xenon atoms were assigned a fixed thermal factor equal to the average thermal factor $<B_W>$ of the crystallographically refined water molecules. The xenon occupancy factor was adjusted periodically, so that its B remains within a few Å^2 equal to the recalculated $<B_W>$. So, there is a constant adjustment of the occupancy factor. The final stages of the refinements were carried out using the stereochemically restrained least-squares minimization method with the PROLSQ program.[19] The data given in Table I represent only a brief summary of these refinements. The full details are reported in

†For simplicity and clarity, the following short names will be used hereafter: elastase, subtilisin, cutinase, collagenase, lysozyme, P64k, urate-oxidase, CytB, and RXR. COMP is an abbreviation for cartilage oligomeric matrix protein.

‡A detailed description of the pressure cell and accessories can be found at the Internet site http://www.lure.u-psud.fr/WWW_ROOT/DOCUMENTS/lure/sections/xenon/xenon.html

TABLE I. Xenon-Protein Complexes: Summary of Structural Data*

Protein	No. of residues	Xe gas pressure (bar)	No. of sites	Refinement of Xe complexes: Final R-factor (%)	Refinement of Xe complexes: High-resolution limit (Å)	Refinement of Xe complexes: No. of reflections used
Elastase	240	8	1	18.3	2.2	10,937
Subtilisin	275	12	1	19.4	2.08	9,665
Collagenase	2 × 230	12	2 × 1	19.7	2.53	17,432
Cutinase (wild-type)	196	12	1	n.r.	—	—
Cutinase (S120A)	196	12	1	n.r.	—	—
Urate-oxidase	4 × 301	8	4 × 1	21.0	2.3	18,523
P64k	2 × 481	13	2 × 2	n.r.	—	—
Lysozyme	129	12	4	16.8	2.1	6,521
RXR	2 × 238	20	2 × 2	n.r.	—	—
CytB	2 × 259	10	1	n.r.	—	—

*The refined structures have been deposited with the Protein Data Bank (n.r. = not refined).

the header of each deposited file within the Protein Data Bank, Brookhaven.

For P64k, the xenon complex was used as a heavy-atom derivative for the resolution of the crystal structure,[33] and during the phasing process, the xenon sites were refined with the program MLPHARE.[43] Strictly speaking, however, the diffraction data were not on an absolute scale, so that the reported occupancies of the xenon atoms may not serve for comparisons with sites in other proteins. For cutinase, the xenon atoms were also refined with MLPHARE, but in this case, high-resolution data had been collected (up to 1.6Å), so that the structure factor amplitudes could be put on an absolute scale by a Wilson plot.

Xenon complexes of DMSO reductase[54] and COMP[34] were described in the literature.

Dr. Schindelin (California Institute of Technology, Passadena, CA, USA) has kindly communicated the co-ordinates of the Xe atom in DMSO reductase to us to include the description of this site in Table II.

RESULTS

Table II summarizes the short contacts observed within a range of c.a. 4.5 Å around the xenon atom at each site. If we add the case of the DMSO-reductase,[54] we remark that hydrophobic contacts are dominant (57 over a total of 77). When the size of the cavities is rather small (i.e., in RXR), it can accommodate only one single atom in a well-ordered position. The observed electron density in the difference-Fourier maps, corresponding to the xenon atom, usually has the shape of a sphere or ellipsoid. However, when the cavity is much larger than the volume of a single xenon or krypton atom, disordered positions are observed as in mutants of phage T4 lysozyme.[45]

The various binding cavities corresponding to the data gathered in Table II are depicted in Figures 1 to 7. The most frequent side chains in interaction are from Ala (6), Leu (21), Val (11), Ile (9), or Phe (5) residues, or Ser, in the special case of serine proteinases.

DISCUSSION

One of the most puzzling features of xenon binding to proteins is the large diversity of the binding sites. Xenon binds to intra- as well as to inter-molecular sites, to closed inaccessible cavities, as well as to exposed pockets and even into channel-pores. The binding sites may be lined up exclusively by aliphatic residues, or they also may include aromatic, or, polar groups. Xenon may bind into void sites, or replace existing water molecules. Below, we have attempted to classify the various binding sites into subsets according to their common features.

Definition

A *cavity* is a region in a protein that is not occupied by protein atoms and that is entirely closed off by the protein. By definition, a cavity is *inaccessible* from the outside, in the static description of a crystal structure. In contrast, a *pocket* (sometimes called *surface invagination*) is connected (*accessible*) to the outside. A pocket would only become a closed cavity if the atomic radii of the protein atoms that delimit the cavity were to be increased. In order to test whether binding sites are cavities or pockets, molecular surfaces were calculated with the Connolly algorithm.[10] Cavities are delimited by closed surface patches that have no connection to the outer molecular surface.

It is well known that the atoms in the interior of protein molecules are densely packed, and that these regions are more similar to solids than to organic liquids. Calculations presented by Klapper[22] have estimated that there is twice as much free volume distributed throughout simple organic liquids than in proteins. However, in proteins, the free volume does not need to be distributed randomly (as in liquids), and empty intramolecular cavities exist in numerous proteins.[21,23] These cavities exist at the

TABLE II. Closest Contacts (Å) Around the Xenon (or Krypton) Atoms in the Various Binding Sites, Within a Sphere of 4.5–4.7 Å

Protein	Atom	Atom (resid.)	Dist.	Atom (resid.)	Dist.	B/Occ*
Elastase	Xe	Oγ(S195)	3.40	O(C191)	3.71	22.6/0.81
		Cγ2(V216)	3.89	Cγ2(T213)	4.0	
Elastase	Kr	Oγ(S195)	3.28	O(C191)	3.90	18.9/0.49†
		Cγ2(V216)	3.75	Cγ2(T213)	3.85	
Subtilisin	Xe	Oγ(S221)	4.03	Cβ(A152)	3.94	29.3/0.71
		Cα(G154)	3.92	Nδ2(N155)	3.73	
		Oγ2(T220)	4.11	Cα(L126)	3.99	
		Ow(310)	4.43			
Cutinase (native)	Xe	Oγ(S120)	4.24	Nδ2(N84)	3.33	19.1/0.81‡
		Cγ2(T150)	4.19	Cγ1(V177)	4.17	
		Cδ2(L182)	3.99			
Collagenase	Xe	Subunit-A:		Subunit B:		
		Oγ(S195a)	3.67	Oγ(S195b)	3.51	28.1/0.95 (in site A)
		Cγ2(V216a)	3.98	Cγ2(V216b)	4.01	
		Cγ1(V213a)	4.22	Cγ1(V213b)	4.33	
		Oε1(Q37b)	3.76	Oε1(Q37a)	4.0	31.8/0.80 (in site B)
		O(S214a)	4.53	O(S214b)	4.58	
		C(C191a)	4.29	C(C191b)	4.27	
		Ow(234)	3.87	Ow(552)	4.40	
Urate-oxidase	Xe	Cδ2(L178)	4.33	Cγ2(T180)	4.79	30.5/0.85
		Cδ1(F219)	4.12	Cγ1(V227)	4.14	
		Cγ2(T230)	3.99	Cδ2(L252)	3.68	
P64k	Xe					
Site #1		Cδ2(L323)	3.42	Cβ(A176)	3.88	22.5/0.55‡
		Cβ(V319)	4.59	O(L172)	3.66	
		Cd1(I179)	4.60	Cδ2(L202)	3.81	
		Cβ(N175)	4.00	Cγ(L172)	4.57	
Site #2		Cδ(L327)	3.60	Cζ(F356)	2.65	30.5/0.40‡
		C(W348)	3.70	Cε(R355)	3.66	
		Oγ1(T318)	3.25	N(L314)	3.45	
Lysozyme	Kr	Cγ2(V92)	3.12	Cδ1(L56)	3.38	19/0.49
		Cγ2(I55)	3.46	Cδ1(I88)	4.16	
		Cε(M12)	3.55	Cβ(S91)	3.89	
Lysozyme	Xe					
Site #1		Oγ1(T43)	3.65	Cβ(T43)	4.00	36.2/0.33
(intermolecular)		N(R45)	4.25	N(N44)	4.36	
		Cγ2(T51)	4.55	Nh2(R68)	4.49	
		Ow(20)	4.50			
		+ symmetry-related				
Site #2		Cγ2(V92)	3.18	Cδ1(L56)	3.33	32.9/0.28
(intramolecular)		Cγ2(I55)	3.51	Cδ1(I88)	4.19	
		Cε(M12)	3.54	Cβ(S91)	3.91	
Site #3		Cα(I58)	4.30	Nε1(W108)	3.75	36.0/0.1
(intramolecular)		O(Q57)	4.03	Cδ1(W1080)	3.96	
		Cδ1(I98)	4.30	O(A107)	3.78	
CytB	Xe	Cδ1(L33)	3.4	Cδ1(I54)	4.2	n.r.¶
(intermolecular)		Cδ1(I233)	4.7	Cζ(F237)	3.9	
		+ symmetry-related				
RXR	Xe					
Site #1		Cγ1(V332)	4.09	O(S336)	4.33	n.r.
		Cα(A337)	3.25	Cβ(A340)	3.36	
		Cγ2(V342)	3.41	O(K440)	3.69	
		Cδ2(L441)	4.23	Cβ(D444)	3.94	
Site #2		Cδ1(L370)	2.92	Cβ(R421)	4.30	n.r.
		Cδ1(L425)	3.78	Cδ1(L422)	4.62	
DMSO-reductase	Xe	Cζ(F110)	3.23	Cβ(A428)	3.58	n.r.
		Cγ1(V402)	3.56	Cε(M405)	4.05	
		Cδ2(L406)	4.21	Cδ2(L452)	4.23	
		Ch2(W449)	4.29			

*Occ = occupancy factor, B = temperature factor (refined, Å^2) for Xe or Kr. Refined using PROLSQ.[19] Standard deviations are within 2 Å^2 for B factor and ±0.1 for Occ.

†Refined with program SHARP.[27]

‡Refined using program MLPHARE[43] after absolute scaling of the data.

¶n.r. = not refined.

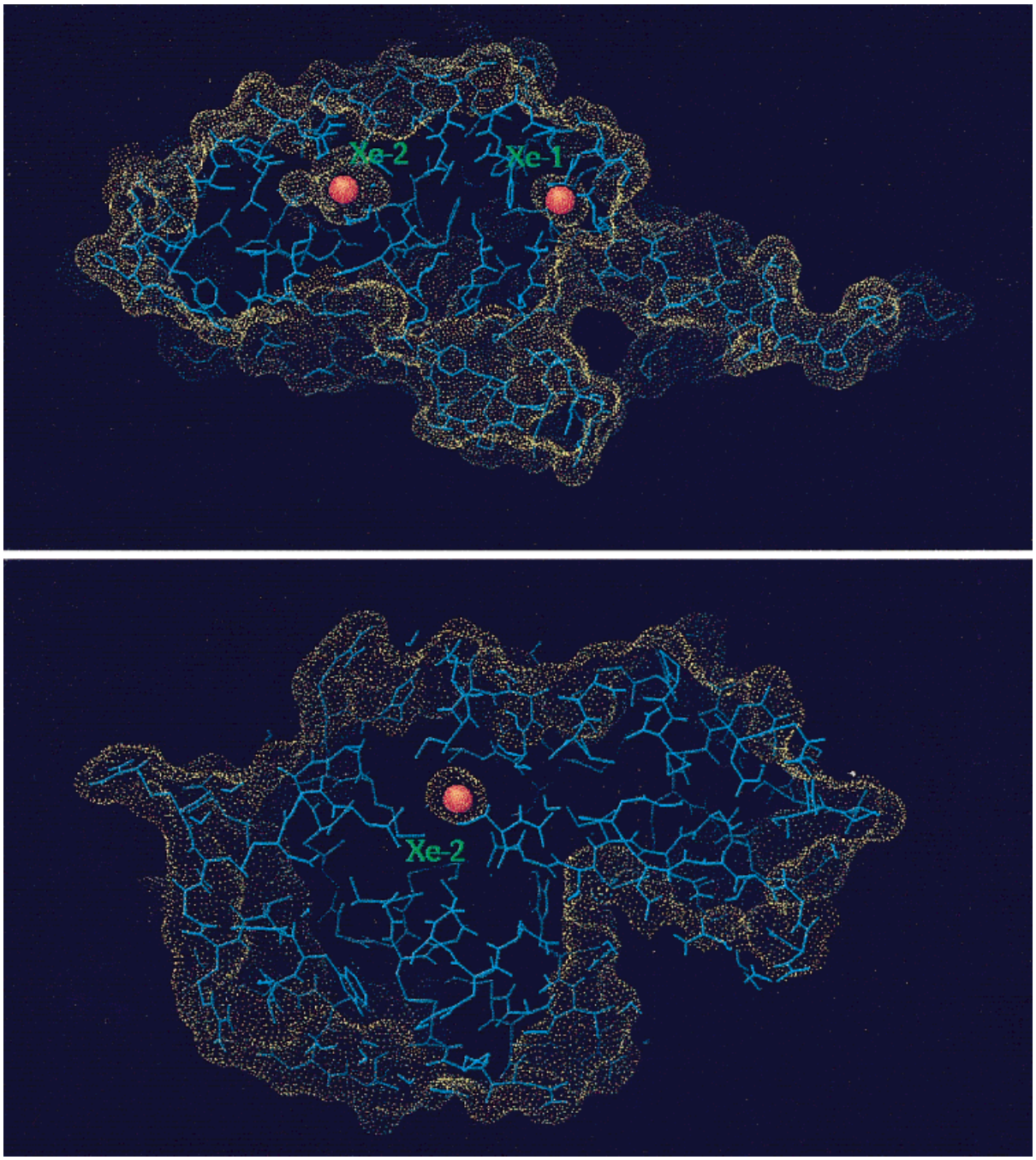

Fig. 1. View of the binding sites of xenon atoms in hen egg lysozyme (**lower**) and RXR (**upper**). The accessible surfaces are calculated by the method of Connolly.[10]

expense of considerable cost in free energy, so that it is unlikely that they are mere packing defects. The hypothesis that cavities are important for the conformational flexibility of protein molecules is supported by the characteristics of xenon binding to myoglobin. Tilton et al.[66] have observed an overall reduction in temperature factors upon xenon binding. This effect 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 xenon binding.[79] Binding of xenon to myoglobin cavities also affects the functionality of the protein in a rather drastic way.[61]

All these observations are evidences of the importance of intramolecular cavities for the dynamics

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