Low-resolution detergent tracing in protein crystals using xenon or krypton to enhance X-ray contrast

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Xenon and krypton show different solubilities in polar *versus* apolar solvents. Therefore, these noble gases should accumulate in apolar regions of protein crystals. Specifically, they should accumulate in lipid and detergent solvent regions within crystals of membrane proteins, which can be used as a basis for contrast-variation experiments to distinguish such apolar solvent regions from the aqueous phase by a low-resolution X-ray diffraction experiment. This possibility was explored with the OmpF porin, one of the general diffusion pores of the *Escerichia coli* outer membrane. Trigonal crystals were exposed to elevated pressures of the two noble gases (up to 10^7 Pa) for several minutes and subsequently flash-cooled to liquid-nitrogen temperatures. Both rare gases bind to a number of 'specific' sites, which can be classified as 'typical' noble-gas binding sites. Compared with a representative water-soluble protein, they are however much more abundant in OmpF. In addition, a very large number of weakly populated sites are observed which accumulate in the region of the 'detergent belt' for crystals exposed to xenon. After application of a Fourier-filtering protocol, low-resolution images of the detergent belt can be obtained. The resulting maps are similar to maps obtained from low-resolution neutron diffraction experiments on contrast-matched crystals.

1. Introduction

A typical, albeit insufficient, prerequisite for the crystallization of membrane proteins is their solubilization by treatment with a mild detergent (Ostermeier & Michel, 1997; Zulauf, 1985). Once crystallized, the detergent is usually still present in the crystal, where it is responsible for stabilizing the protein and shielding its hydrophobic surface against the aqueous phase. Knowledge of the distribution of the detergent in such crystals provides insight into how the protein is attached to or interacts with the membrane and how the detergent is involved in the crystallization process (Timmins *et al.*, 1994). Functionally relevant regions of lipids and/or detergents have also been observed in crystals of lipases (Hermoso *et al.*, 1997).

While membrane-protein crystals often diffract to high resolution and show a correspondingly well ordered three-dimensional structure, regions occupied by lipid or detergent are always highly disordered. It is difficult to locate or identify such disordered regions by standard protein X-ray crystallography since their structural information is only contained in (often ignored or neglected) low-order reflections. Moreover, the contrast between lipid/detergent and (equally disordered) aqueous solvent is small. Specific techniques have to be

employed to enhance the contrast between detergent and water in the crystal and to collect low-resolution diffraction data. Such experiments have been the realm of neutron crystallography (Timmins, 1995a), which exploits the large difference in scattering-length density between hydrogen and deuterium. With neutrons, contrast variation is possible by varying the H_2O/D_2O ratio of the solvent or by using appropriately deuterium-labelled detergents (Hermoso et al., 1997; Pebay-Peyroula et al., 1995; Penel et al., 1998; Roth et al., 1989, 1991; Timmins et al., 1992, 1994; Timmins, 1995a,b; Timmins & Zaccai, 1988; Zulauf et al., 1986). These elegant experiments have yielded spectacular insight into the distribution of lipids and detergents in a variety of systems, but they suffer from the low flux of available neutron sources and from difficulties in obtaining isotopically labelled samples.

Macromolecular X-ray crystallography started to exploit contrast-variation techniques at a very early stage (Bragg & Perutz, 1952). Indeed, it was shown repeatedly that the solvent density in protein crystals can be changed by the addition of organic compounds (such as sucrose or glycerol) or ions (e.g. caesium, rubidium, bromide, selenate etc.) to the mother liquor (Carter et al., 1990; Fourme et al., 1995; Sigler & Blow, 1964) and that this can be exploited to extract information about the phases. By and large, however, contrast variation has been the realm of neutron crystallography (Ibel & Stuhrmann, 1975). Moreover, we are not aware of contrast-variation X-ray crystallography being used to distinguish polar from apolar liquid phases in membrane-protein crystals.

Recently, a technique was developed which makes it possible to introduce relatively high concentrations (several hundred millimolar) of xenon and other noble gases into the mother liquor of a protein crystal, often without affecting the crystal's integrity (Sauer et al., 1997; Soltis et al., 1997). The technique involves the exposure of the protein crystal to pressurized gas and the subsequent rapid flash-freezing to liquid-nitrogen temperature. Its original objective was to obtain isomorphous heavy-atom derivatives to solve the crystallographic phase problem by MIR, SIR or MAD techniques. Since the interaction of xenon with the protein is confined to weak dispersion forces, the high interstitial xenon concentration created by exposure to pressurized gas very often has no adverse effects on the crystal packing.

Xenon is a 'soft' noble gas: the diameter of an Xe atom is about 4.2 Å and its atomic polarizability is $\alpha = 4.01 \times 10^{24}$ cm^3. These properties are similar to those of a methane molecule, which makes xenon with its 54 electrons an ideal hydrophobic probe for X-ray crystallography (Prangé et al., 1998). In fact, a strong preference of xenon for specific apolar sites has emerged from crystallographic experiments (Prangé et al., 1998) as well as from molecular-dynamics simulations (Kocher et al., 1996; Prevost et al., 1996; Tilton et al., 1986). It is well known that the solubility of xenon depends strongly on the polarity of the solvent, which manifests itself in a large difference between xenon solubility in water and n-octane (Kennan & Pollack, 1988; Pollack, 1991). Therefore, one might expect that xenon also dissolves more readily in the lipid or detergent phase surrounding membrane proteins than in the

aqueous phase of the mother liquor. Imaging the location of such disordered hydrophobic detergent phases within crystals of membrane proteins should therefore be possible by introducing an electron-density contrast as a result of exposing the crystal to pressurized xenon. Using bulk-solubility data (Pollack, 1991) and simple approximations (e.g. the validity of Henry's law), one can estimate that a xenon pressure of 10^5 Pa introduces a contrast of $\Delta\rho \simeq 5 \times 10^{-3}$ e Å^{-3}, which is about 1% of the mean electron density of a protein. While it is unclear whether the solubility increases linearly with the pressure at high pressures (Kennan & Pollack, 1990), it is at least plausible to assume an increase of contrast with increasing pressure well beyond ambient conditions. Although beyond 2×10^6 Pa xenon clathrates are formed in aqueous solutions (De Forcrand, 1925), clathrate formation appears to be impeded by most constituents of mother liquors of protein crystals and poses no practical limitation for flash-freezing experiments up to at least 10^7 Pa (Sauer et al., 1997).

Exploratory experiments for its use as a crystallographic heavy atom (Cohen et al., 2001) as well as a probe for hydrophobic protein regions have also been reported for krypton (Alvarez & Prini, 1992; Prangé et al., 1998; Schiltz et al., 1997). Krypton is more inert and less soluble than xenon and its smaller size allows it to occupy smaller cavities, but it interacts less strongly with the protein owing to its lower polarizability (Schiltz et al., 1997). While its reduced solubility may result in a correspondingly smaller contrast-variation signal, the position of its K absorption edge ($\sim$0.84 Å) invites multiple anomalous dispersion experiments (Cohen et al., 2001). For the same reason, krypton might also be a candidate for the MASC method (Fourme et al., 1995) to phase low-order reflections.

In the present communication, we describe contrast-variation experiments with xenon and krypton on the OmpF porin, one of the general diffusion pores of the E. coli outer membrane (Schirmer, 1998). OmpF porin is responsible for the uptake of nutrition and the disposal of metabolic waste products. As shown by X-ray crystallography (Cowan et al., 1992), the monomeric protein consists of 16 antiparallel

Table 1
Summary of data sets collected from OmpF.

	Nat2	Xe-005a	Xe-050a	Xe-100a	Kr-050a
Pressure (Pa)	0	5×10^5	5×10^6	1×10^7	5×10^6
Beamline	Home	Home	Home	Home	Trieste
Wavelength (Å)	1.5418	1.5418	1.5418	1.5418	0.88
Resolution (Å)	51–3.61	30.7–3.6	46–3.6	14–2.5	23.3–2.2
Completeness (%)	95	98	99	91	98
Redundancy	4.5	2.1	4.9	4.7	7.1
$I/\sigma(I)$ high	3.1	2.7	2.6	2.0	1.4
$I/\sigma(I)$ low	9.6	3.5	6.9	11.0	13.1
R_{sym} (%)	12.2	13.3	16.4	10.8	6.2
R_{anom} (%)	8.6	10.2	10.3	9.2	2.6
Space group	$P321$	$P321$	$P321$	$P321$	$P321$
$a = b$ (Å)	117.31	116.98	116.64	116.84	116.90
c (Å)	52.01	51.45	51.69	51.66	51.35
Final R (%)	22.7	23.0	26.9	31.1	28.8

† $R_{sym} = \sum |I - \langle I \rangle| / \langle I \rangle$. ‡ $R_{anom} = \sum(|\langle I^+ \rangle - \langle I^- \rangle|)/\sum(\langle I^+ \rangle + \langle I^- \rangle)$.

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β-strands forming a slightly ovoid barrel structure. In the membrane it occurs as a homotrimer. The loops which connect the barrel's β-strands are short and regular towards the periplasm ('smooth end') and long, irregular but tightly packed towards the cell exterior ('rough end'). One long loop ('pore constriction loop') contains a short α-helical segment and extends inside the hollow barrel. This loop is believed to control the fine-tuning of the channel characteristics (Huang & Igo, 1996; Phale *et al.*, 1997; Van Gelder *et al.*, 1997).

At the perimeter of the trimer, a hydrophobic band composed mainly of small aliphatic residues can be discerned (Cowan *et al.*, 1992). This would contact the hydrophobic core of the membrane *in vivo*. In the crystals, this part of the surface is covered by detergent molecules as evidenced by low-resolution neutron diffraction studies on tetragonal (Pebay-Peyroula *et al.*, 1995) and trigonal (Penel *et al.*, 1998) OmpF crystals. These contrast-variation studies also allowed discrimination of the tail and head regions of the amphiphiles. For the tetragonal crystals, the detergent forms a belt surrounding each trimer and is also involved in crystal contacts between trimers. In trigonal OmpF crystals, trimers are arranged in layers perpendicular to the crystallographic triad, leaving solvent-filled channels running parallel to this axis. Within each layer, the trimeric protein is arranged in a pseudo-hexagonal pattern. The detergent is at the inside of such hexagons of trimers, where it surrounds the major solvent channel in a reverse-micelle like fashion. The height of the detergent belt is about 25 Å in both crystal forms, and two 'aromatic girdles' consisting of phenylalanine and tyrosine residues demarcate the detergent domain. These girdles are a characteristic for β-barrel and α-helical membrane proteins (Pautsch & Schulz, 1998) and probably help to anchor the protein in the lipid bilayer.

2. Experimental

Crystals were grown from a buffer containing 0.05 *M* Tris–HCl pH 9.8 and 0.6%(*w/w*) *n*-octyl-2-hydroxyethylsulfoxide and 0.1% octyl POE as detergent, 0.7 *M* MgCl$_2$ and 6%(*w/w*) PEG 2000, the concentration of which was raised to about 10% by microdialysis (Pauptit *et al.*, 1991).

2.1. Preparation of Xe and Kr derivatives

Xenon and krypton derivatives of OmpF porin crystals were obtained with the locally constructed high-pressure device described previously (Sauer *et al.*, 1997). Crystals of typical volumes between 2×10^{-3} and 2×10^{-2} mm^3 were transferred from their mother liquor into a cryosolution [same composition as mother liquor plus 10% PEG 200 and 15%(*v/v*) glycerol] and mounted within a cryoloop (Teng, 1990). Noble-gas
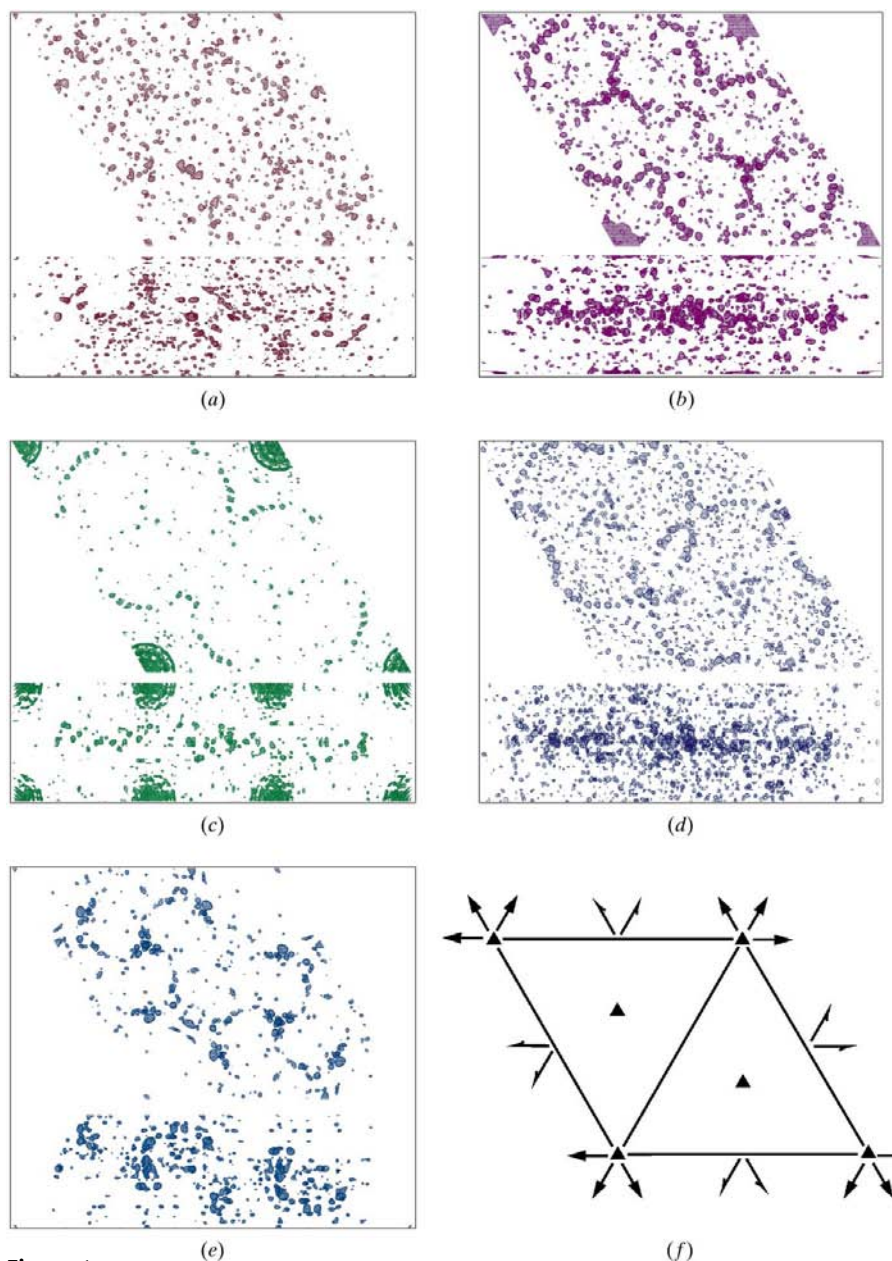

Figure 1
Initial Fourier maps from OmpF after subjection to xenon and krypton. In each of the panels, the density is projected along (upper part of the panel) and perpendicular (lower part of the panel) to the crystallographic threefold axis. (*a*) Difference density map after exposure to xenon at 5×10^5 Pa, contoured at 3.0σ, (*b*) 5×10^6 Pa, 2.5σ, (*c*) 1×10^7 Pa, 3.0σ. (*d*) Density maps calculated with the observed anomalous differences from OmpF after subjection to 5×10^6 Pa xenon-gas pressure, 2.5σ contours. (*e*) Difference-density maps after subjection to 5×10^6 Pa krypton gas, 2.8σ contours. (*f*) The crystallographic symmetry elements of the upper part of each panel.

pressures ranging from 5×10^5 to 1×10^7 Pa (see Table 1) were applied to the crystals for 3–4 min. Immediately after pressure release crystals were flash-frozen (within a few seconds) by dumping into liquid nitrogen.

2.2. Collection of diffraction data

Following flash-freezing, diffraction data were collected either in-house (Siemens rotating-anode generator, Cu $K\alpha$ radiation, mirror collimation system, MAR300 image-plate goniometer) or at the diffraction beamline at Sinchrotrone Trieste, Italy.

Care was taken to collect low-resolution data to about 50 Å (see Table 1), using long crystal-to-detector distances (typically 350 mm at our home source) and, where available, a helium path between the crystal and the detector. Crystals diffracted on average to 2.5 Å, but anisotropy reduced the

high-resolution limit in the direction of the c^* axis. This effect increased with gas pressure. A native data set was also collected in-house, using essentially the same data-collection parameters as for the derivatives. Several data sets with different experimental conditions were collected, as summarized in Table 1.

2.3. Data processing

2.3.1. Data reduction. Reflection intensities were integrated with the program *DENZO* (Otwinowski, 1990). The post-refinement was performed with *SCALEPACK* and refined values therefrom were used to reintegrate the images, which were then scaled using *SCALA*, applying local scaling (Collaborative Computational Project, Number 4, 1994). No *B*-factor refinement was applied, because radiation damage
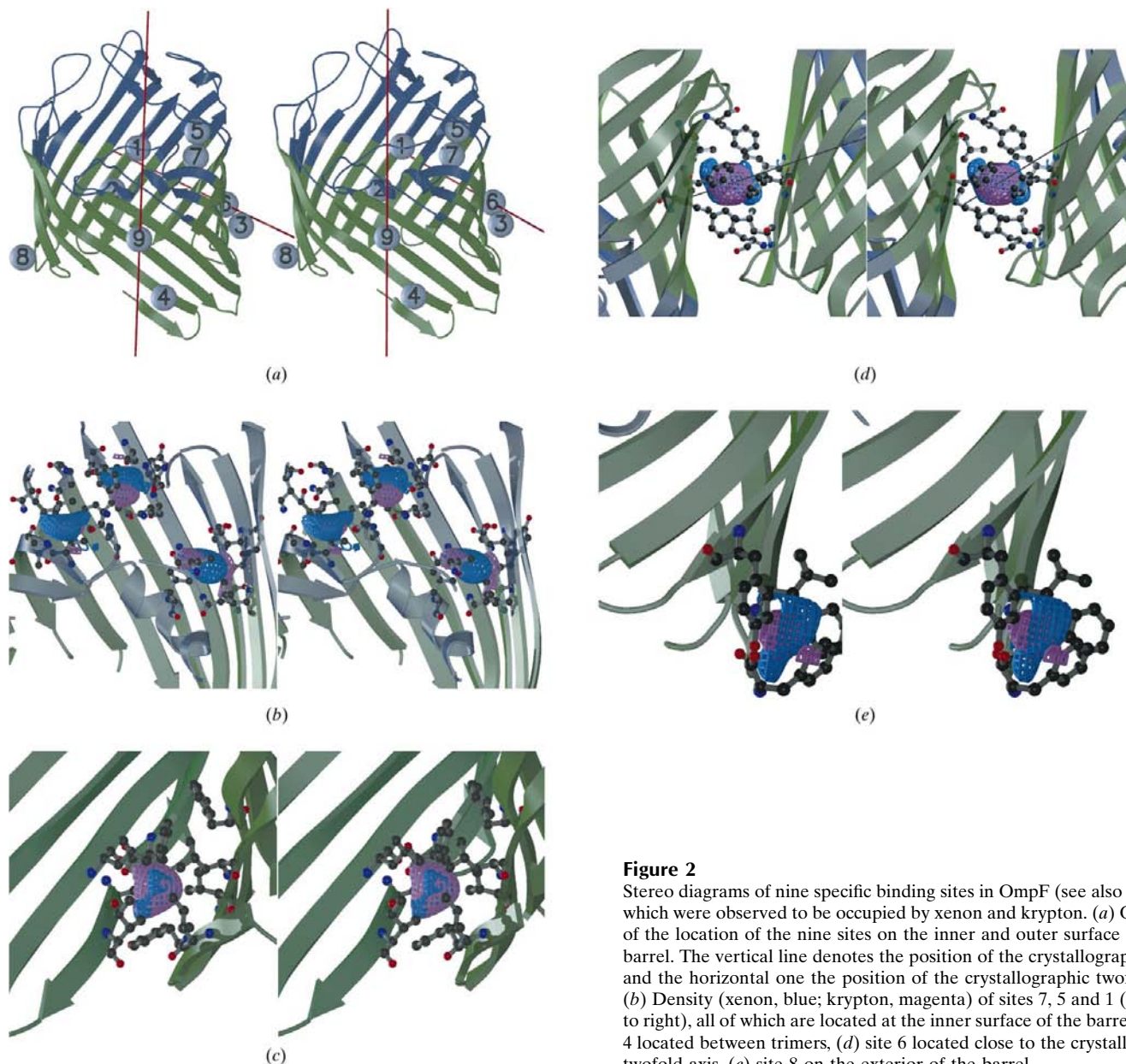
Figure 2
Stereo diagrams of nine specific binding sites in OmpF (see also Table 2), which were observed to be occupied by xenon and krypton. (*a*) Overview of the location of the nine sites on the inner and outer surface of the β-barrel. The vertical line denotes the position of the crystallographic triad and the horizontal one the position of the crystallographic twofold axis. (*b*) Density (xenon, blue; krypton, magenta) of sites 7, 5 and 1 (from left to right), all of which are located at the inner surface of the barrel, (*c*) site 4 located between trimers, (*d*) site 6 located close to the crystallographic twofold axis, (*e*) site 8 on the exterior of the barrel.

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Site No.	1	2	3	4	5	6	7	8	9
x, y, z coordinates (Å)	1.9, 34.9, 37.5	−2.4, 32.6, 8.4	20.9, 47.0, 26.2	5.1, 67.4, 18.4	12.4, 40.8, 43.3	19.0, 32.9, 25.9	11.7, 49.1, 40.3	−19.8, 42.3, 18.2	0.0, 67.5, 29.9
Surrounding residues	T122, L258, A215, D256, L109, N230, G232, Y231	A228, L227, A229, L218, K219, Y111, T112, V260	S95, F96, T138, Y139, R140	I3, Y4, A1, V11, L13	F128, F129, G192, A193, A194, A211, E212, Q213	F144, L151, F153, I178, F′144, L′15, F′153, I′178	F128, V133, D134, V136, L158, A170	F265, F267, G268, L269, Y301	L43, W61, F65, L′43, W′6, 1F′65, L″43, W″61, F″65
Hydropathy	−1.1	1.0	−0.1	2.5	0.1	3.6	1.6	1.9	3.8

was found to be negligible and data were collected only to relatively low resolution.

2.3.2. Difference maps, phasing. Phasing was based on the native crystal structure of OmpF porin (Cowan *et al.*, 1992). A protein model devoid of detergent and water molecules was refined against each data set using a rigid-body and subsequently a positional refinement procedure as implemented in program *X-PLOR* (Brünger, 1992). The resulting protein model was very similar to the native OmpF crystal structure for all xenon pressures (r.m.s.d. < 1 Å). No refinement of individual B factors was attempted in view of the low resolution. Phases for subsequent difference maps were calculated from the refinement of the protein part of the structure against the native data.

Difference maps using the above phases were calculated for the overlapping resolution range between native and derivative data sets. For the calculation of phases from the native data set, a bulk-solvent correction was applied which was smoothed with a Gaussian ($B = 125$ Å^2) to prevent artefacts near the protein envelope.

2.3.3. Model building. Pseudo-sites were distributed within the difference-density map using the program *MAPMAN* (Kleywegt & Jones, 1996). The threshold limit for the distribution of sites was chosen close to the noise level. Thus, multiple sites may have been assigned to regions of high difference density, but the low threshold value assures that regions of weak density were also accounted for (Kleywegt & Read, 1997; Kleywegt & Jones, 1996). The σ value of each pseudo-site was then plotted against the hydropathy value of the corresponding site. To assign a hydropathy value to a location, the following formula was used:

$$\langle \mathrm{hp} \rangle = \sum_{i=1}^{\mathrm{alldist}} \mathrm{hp}_i \cdot d_i^{-2} / \sum_{i=1}^{\mathrm{alldist}} d_i^{-2}.$$

$\langle \mathrm{hp} \rangle$ is the mean hydropathy for the site and d_i is the distance to the closest atom of residue i with hydropathy hp_i (Gilbert, 1992; Kellogg & Abraham, 1992). Hydropathy values were used as implemented in the program *O* (Jones *et al.*, 1991). The corresponding plot of σ *versus* hydropathy is shown in Fig. 5 for the pseudo-sites obtained from the 5×10^6 Pa Xe experiment (Xe-050a) and was used to determine a σ cutoff level in such a way that the highest peak in the region of negative hydropathy was just below the cutoff. The remaining above-threshold sites were then each populated with a fully

occupied Xe atom with $B = 20$ Å^2 and a structure-factor calculation was performed with these xenon positions. A density map was subsequently computed by Fourier-summation of all $F_c(hkl)$ within a resolution shell between 100 and 10 Å. The resulting map is shown in Fig. 3.

3. Results

We performed a series of experiments with trigonal crystals of the *E. coli* membrane protein OmpF, which were subjected to xenon and krypton gas at elevated pressures and were subsequently flash-frozen by dumping into liquid nitrogen. Pressures of up to 10^7 Pa and exposure times around 3 min were chosen. The frozen crystals were subsequently mounted on a diffractometer under a stream of cold nitrogen (~100 K) and X-ray diffraction data were collected. A selection of data sets collected under a variety of experimental conditions is summarized in Table 1. The structures were subsequently solved by molecular replacement, using the protein moiety of the crystal structure of native OmpF as a search model, and refined by rigid-body least-squares methods. Using the phases from this 'protein-only' model, difference-Fourier maps between native and gas-absorbed crystals were calculated. Examples of such initial difference-density maps are shown in Figs. 1(*a*), 1(*b*) and 1(*c*) for xenon, and Fig. 1(*e*) for krypton. In addition, anomalous maps were computed for Xe-exposed crystals, using the observed anomalous differences as amplitudes of the Fourier summation and the phases obtained from the above 'protein-only' model. Projections of such a map are shown in Fig. 1(*d*).

Irrespective of the gas pressure, the maps obtained from crystals treated with xenon invariably show numerous patches of difference density which appear to cluster at the envelope around the porin trimers when viewed along the crystallographic triad. When viewed perpendicular to the crystallographic triad, accumulation of density appears roughly in the middle of each protein barrel (Figs. 1*a* to 1*d*). Maps obtained from Kr-treated crystals show a somewhat different appearance. Overall, the krypton difference signal is weaker, indicative of the overall lower affinity of krypton. In addition, the signal appears to be confined to fewer sites, which unlike the Xe density do not cluster around the centre of the hydrophobic outer surface of the β-barrels (Fig. 1*e*).

apolar residues such as Leu, Val, Ile or Phe, have been observed in many other proteins (Prangé *et al.*, 1998). The hydrophobic sites at the interface between two or three OmpF molecules (sites 4, 6 and 9) correlate with xenon sites occasionally observed between domains or subunits of a protein. An example of such a binding site was recently observed in the crystal structure of glutamate mutase (Reitzer *et al.*, 1999; Sauer, 2001). Compared with other (water-soluble) proteins, OmpF is not outstanding with regard to the nature of the 'buried' binding sites, but rather with regard to their large number.

The observation of specific binding sites for xenon or krypton in OmpF was not the main objective of the present study. For this reason, our diffraction experiments were not optimized to observe such sites, *e.g.* we only collected diffraction data to relatively low resolutions (see Table 1). The data therefore do not permit the refinement of occupancies for these sites, which were only estimated on the basis of the

signal height. However, observation of such sites provides insight into the arrangement of cavities within the protein. Specific binding is required in order to solve the protein structure by isomorphous replacement (MIR) or multiple anomalous dispersion methods (MAD). Typically, the occupancy of these sites is almost independent of the rare-gas pressure. In fact, to observe good occupancy of these 'specific' sites, pressures well below 5×10^6 Pa are sufficient and, at least in the case of xenon, preferable to higher pressures, avoiding the noise of numerous sites of low occupancy. Although the occupancy of these sites appears to be higher for sites with apolar environment, some of the specific sites in OmpF are at least moderately polar. It has been noted previously from xenon derivatives of other proteins that
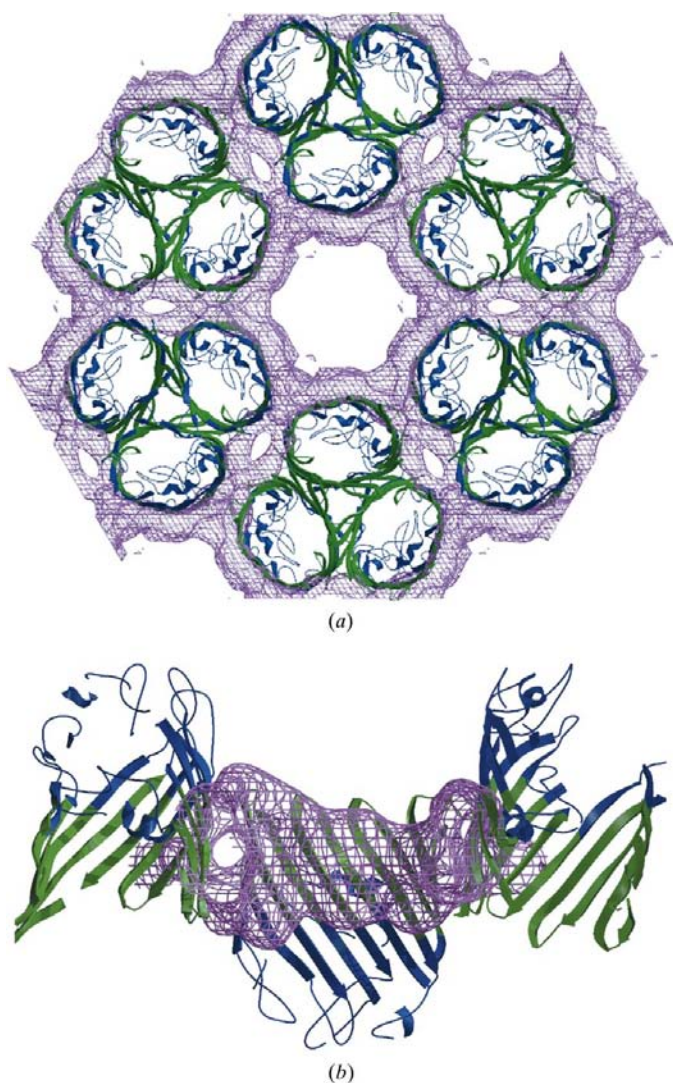
Figure 3
The smoothed low-resolution xenon-enhanced X-ray density map, as obtained from the 5×10^6 Pa experiment. (*a*) View down and (*b*) perpendicular to the crystallographic threefold axis.
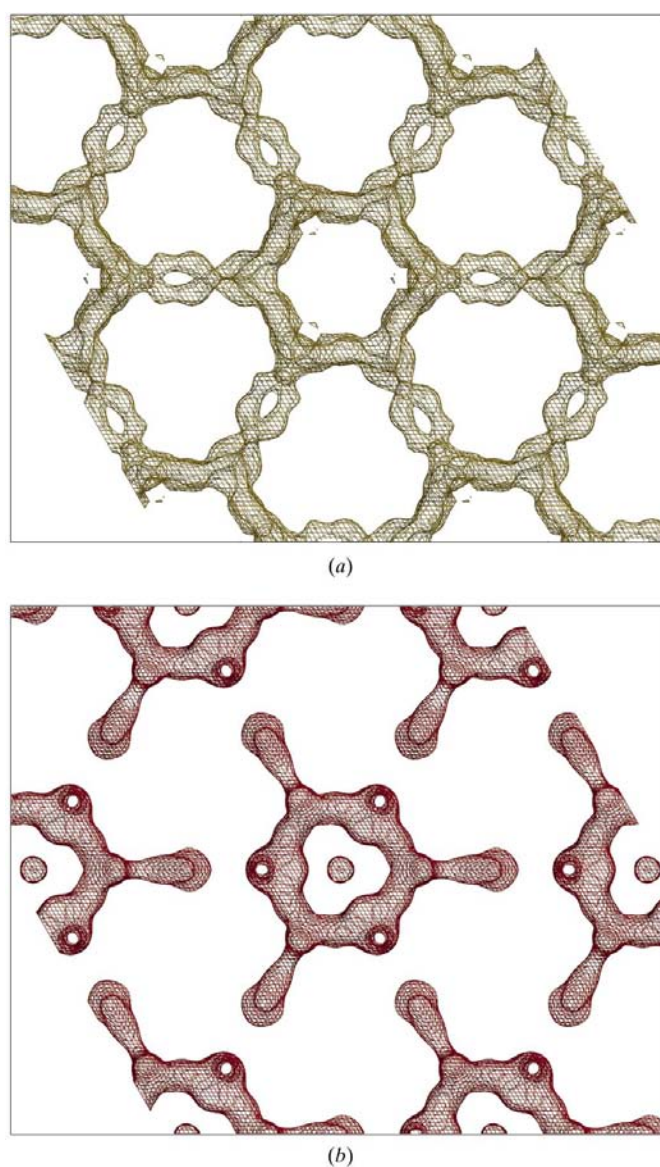

Figure 4
Comparison of low-resolution detergent-tracing maps as obtained by the 5×10^6 Pa xenon-enhanced X-ray (*a*) and neutron (*b*) diffraction, projected down the crystallographic threefold axis of trigonal Ompf crystals.

specific binding sites are not confined to a hydrophobic vicinity (Schiltz et al., 1995)

4.2. Xe images detergent regions of OmpF porin

The 'open' sites, which interact with protein side chains pointing into the solvent or detergent region, are much more abundant than the 'buried' sites, but show much lower peak heights owing to partial occupancy. They are populated more efficiently by xenon than by krypton. In order to improve the visibility of their relatively diffuse density, to suppress the inherent noise (originating from experimental errors, lack of isomorphism etc) and to obtain a model comparable with the neutron diffraction results, the map was Fourier-filtered as described in §2 and §3. The resulting maps are shown in Fig. 4.

Evidently, the xenon accumulates in a belt surrounding each trimer. The model which we computed from the difference-densities has a belt with an average width around 25 Å. The belt is confined by two rows of aromatic protein residues (mainly Tyr) surrounding the trimer. The thickness of the belt (i.e. its maximum distance from the protein surface) is up to 14 Å, which exceeds the length of a C_8 alkyl chain.

There are two types of solvent-filled channels running parallel to the crystallographic c axis through trigonal OmpF crystals: larger ones through the origin of the unit cell and smaller ones passing between adjacent trimers. Extensive Xe difference density lines the inner surface of both channels, covering exposed hydrophobic protein surfaces. One can roughly estimate that the volume with increased xenon density in the large channel amounts to approximately 24 000 Å³, which is of the order of 4% of the total cell volume. In the neutron study of trigonal OmpF, the detergent model was found to occupy a much larger volume, as much as 14% of the unit-cell volume including head groups (Penel et al., 1998). Radioactive labelling has indicated a detergent content between 20 and 36% of the cell volume for OmpF crystals (Garavito et al., 1983).
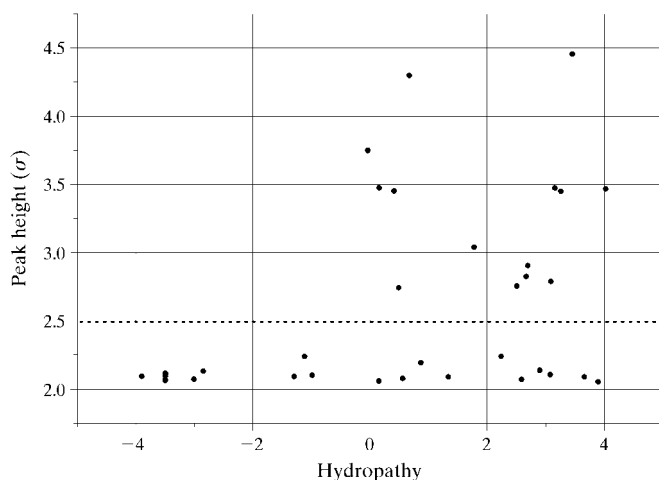
4.3. Neutron versus X-ray imaging

The detergent structure in trigonal OmpF porin crystals was extensively investigated by neutron diffraction (Pebay-Peyroula et al., 1995; Penel et al., 1998). Although the results of this analysis are consistent with the present X-ray study, there are minor discrepancies. Most apparent is the difference in the thickness of the density belt obtained by the two different methods (Fig. 4). Different influences may account for this, but it is difficult to assess their individual contributions.

The most obvious potential cause for discrepancies are differences in the effective crystallographic resolution: while the neutron study was based on data extending to 16 Å, the X-ray data were collected to much higher resolution (3.6 Å or better, see Table 1), but were then Fourier-filtered which effectively reduced the resolution. There are also more subtle methodological differences between the two studies, e.g. in the neutron study the detergent region was modelled by regularly distributing pseudo-sites into the experimental neutron density, which were subsequently shifted to minimize the crystallographic residual. In contrast, the X-ray study involved the distribution of Xe atoms on observed but weakly occupied Xe-binding sites. Such sites are predominantly located on the protein surface near hydrophobic residues and it is plausible that many of these sites are located within the interaction area between detergent tails and protein. Further away from the protein surface, Xe atoms probably intercalate between detergent tails, but are likely to be less visible owing to disorder, which rapidly increases with increasing distance from the protein surface (Fig. 6). Consequently, compared with the neutron contrast variation, xenon gives a stronger contrast signal close to the protein surface.

Figure 5
Plot of the peak heights observed in the 5×10^6 Pa xenon difference map (Fig. 1b) versus the hydropathy of the corresponding site. See text for details of the formula used to compute the hydropathy value. The dotted horizontal line indicates the σ-cutoff threshold.
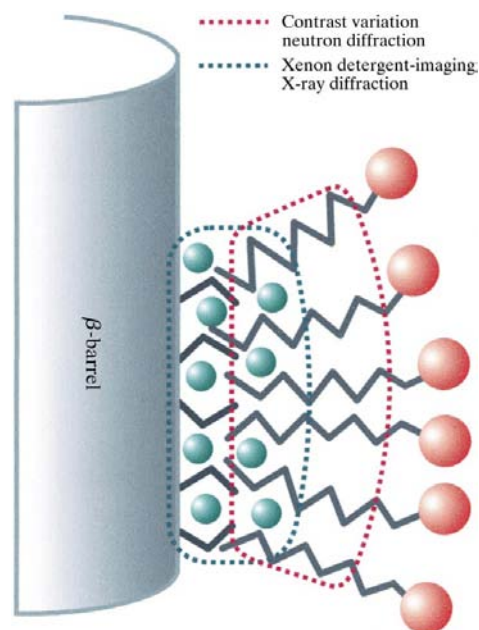

Figure 6
Schematic representation of the reasons for differences in low-resolution detergent-tracing maps as observed by contrast-variation neutron diffraction and by xenon-enhanced X-ray diffraction.

Despite the observation of differently occupied minor sites from one experiment to another, the resulting model after smoothing turns out to be very similar between different experiments. Since the apparent width of the xenon density belt depends somewhat on the resolution employed during back transform, we chose a resolution similar to that of the neutron experiment to obtain comparable results.

4.4. Krypton *versus* xenon sites

Using similar experimental conditions for producing a xenon and a krypton derivative, the Fourier peak density was found to be smaller in the case of krypton. This is not surprising in view of the fact that Kr is less soluble than Xe and produces an inherently smaller signal owing to its smaller number (by 18) of electrons. The pressure required to attain comparable concentrations of both gases in water was estimated to be about 30% higher for krypton than for xenon (Schiltz *et al.*, 1997).

Nevertheless, it is remarkable that little Kr difference density is found at the interface between detergent and protein, while sites inside the protein or at protein crystal contacts show significant occupancies. The highest Kr peak is located in a cavity confined by Leu109, Thr122 and Leu258 and Asp256 (site No. 1; see Table 2 and Fig. 2*b*). This cavity has a volume of 143 Å^3 and emerges when the pore constriction loop folds into the barrel. Xenon also occupies this site, but the highest xenon occupancy occurs at the crystal contact along the twofold axis.

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