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Heavy-atom derivatives in lipidic cubic phases: results on hen egg-white lysozyme tetragonal derivative crystals with Gd-HPDO3A complex

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Gd-HPDO3A, a neutral gadolinium complex, is a good candidate for obtaining heavy-atom-derivative crystals by the lipidic cubic phase crystallization method known to be effective for membrane proteins. Gadolinium-derivative crystals of hen egg-white lysozyme were obtained by co-crystallizing the protein with 100 m*M* Gd-HPDO3A in a monoolein cubic phase. Diffraction data were collected to a resolution of 1.7 Å using Cu $K\alpha$ radiation from a rotating-anode generator. Two binding sites of the gadolinium complex were located from the strong gadolinium anomalous signal. The Gd-atom positions and their refined occupancies were found to be identical to those found in derivative crystals of hen egg-white lysozyme obtained by co-crystallizing the protein with 100 m*M* Gd-HPDO3A using the hanging-drop technique. Moreover, the refined structures are isomorphous. The lipidic cubic phase is not disturbed by the high concentration of Gd-HPDO3A. This experiment demonstrates that a gadolinium complex, Gd-HPDO3A, can be used to obtain derivative crystals by the lipidic cubic phase crystallization method. Further studies with membrane proteins that are known to crystallize in lipidic cubic phases will be undertaken with Gd-HPDO3A and other Gd complexes to test whether derivative crystals with high Gd-site occupancies can be obtained.

1. Introduction

Some proteins, such as membrane proteins, are difficult to solubilize into a homogeneous and monodisperse solution because they possess both hydrophilic and hydrophobic surfaces. The use of detergents (Helenius & Simons, 1975; Tanford & Reynolds, 1976), which form belts that cover the hydrophobic membrane domains, have been employed in order to overcome this difficulty and to make membrane proteins water soluble. Crystallization of such protein–detergent assemblies resulted in the first crystals of membrane proteins (Michel & Oesterhelt, 1980; Garavito & Rosenbusch, 1980); the majority of membrane-protein crystals have been obtained using this method (see http://www.mpibp-frankfurt.mpg.de/michel/public/memprotstruct.html and http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html). Landau & Rosenbusch (1996) have proposed a different approach using the properties of lipidic cubic phases for stabilization and crystallization of the membrane proteins. Using a lipidic cubic phase, formed by curved continuous bilayers of lipid with interconnecting channels filled with water and protein to which precipitant agent is added, they obtained well diffracting bacteriorhodopsin crystals. Crystals grown in monoolein-based cubic phases were used to solve at high resolution the structures of bacteriorhodopsin (Belrhali *et al.*, 1999; Luecke *et al.*, 1999), halorhodopsin (Kolbe *et al.*, 2000) and sensory rhodopsin II (Royant *et al.*, 2001). Chiu *et al.* (2000) have shown the general applicability of the lipidic cubic phase crystallization method to other membrane proteins such as the photosynthetic reaction centres of the purple bacteria *Rhodopseudomonas sphaeroides* and *Rhodobacter viridis* and the light-harvesting complex 2 from *Rhodopseudomonas acidophila*. Crystals of polar proteins, such as lysozyme, have also been obtained in the aqueous compartment of lipidic phases and were isomorphous to those crystallized in aqueous solution (Landau *et al.*, 1997). No attempt has been made to directly produce derivative crystals with lipidic cubic phases.

In a recent paper, Girard, Stelter, Anelli *et al.* (2003) proposed a new class of gadolinium complexes that can be used to obtain high-phasing-power heavy-atom derivative crystals of soluble proteins for anomalous diffraction experiments. The derivative crystals are prepared either by co-crystallization using classical vapour-diffusion techniques or by soaking native crystals in a solution with the same composition as the well solution and to

which the complex has been added at high concentration (100 m*M*). In the present study, we focus on the use of Gd-HPDO3A, one such complex, in order to obtain lysozyme derivative crystals by lipidic cubic phase crystallization (CP derivative). Crystals were obtained by co-crystallizing lysozyme with 100 m*M* Gd-HPDO3A in a monoolein-based lipidic cubic phase. Anomalous diffraction data were collected using Cu $K\alpha$ radiation from a rotating-anode generator. Subsequent refinement of the structure has been performed in order to compare the occupancies of the two Gd-HPDO3A binding sites in the CP-derivative crystals with those of the same binding sites in lysozyme derivative crystals obtained by hanging-drop co-crystallization (HD derivative; Girard *et al.*, 2002).

2. Materials and methods

2.1. Materials

Hen egg-white lysozyme (HEWL) was purchased from Boehringer and was used without any further purification. Lipids were purchased from NuCheck Prep and *Candida rugosa* lipase was purchased from Sigma. Gd-HPDO3A was kindly provided by Bracco Imaging, Milan.

2.2. Crystallization

CP-derivative crystals of HEWL were grown at 293 K using conditions adapted from Landau *et al.* (1997). The lipidic cubic phase was prepared by mixing in a glass tube dry monooleyl-*rac*-glycerol (monoolein, MO) with an aqueous solution containing 40 mg ml^{-1} HEWL, 50 m*M* sodium acetate buffer pH 4.5 and 100 m*M* Gd-HPDO3A.

According to the known phase diagrams of lipid–water systems (Qiu & Caffrey, 2000), the aqueous solution:MO ratio was chosen to be 40%(*w*/*w*) aqueous protein solution *versus* 60% MO in order to form the lipidic cubic phase. In practice, about 5 mg of MO were placed in each glass tube. The tubes were centrifuged at 8800*g* for a few minutes so that the MO was at the bottom of the tubes and the tubes were incubated at 304 K in order to melt the MO. Adding the aqueous solution formed the lipidic cubic phase. The tubes were centrifuged again at 8800*g* in two runs of 15 min. Between the runs, the tubes were rotated by 180° about their long axis. A transparent non-birefringent gel-like material was obtained at the bottom of the tube. The tubes were left at 293 K for a few (2–12) hours before adding a sodium chloride solution as a precipitant to induce crystallization. During the whole process, the tubes were sealed with film. With precipitant concentrations in the range 0.6–0.8 *M*, CP-derivative HEWL crystals of 200 µm in length were obtained.

2.3. Manipulation and freezing

Prior to cryocooling and data collection, crystals have to be removed from the lipidic cubic phase. This was performed by the enzymatic hydrolysis method described by Nollert & Landau (1998). Within a few hours, *C. rugosa* lipase converts the lipidic cubic phase into a two-phase system that consists of two immiscible liquids, water/glycerol solution and oleic acid. Crystals are thus released from the lipidic matrix. In order to maintain the Gd-HPDO3A concentration during the hydrolysis process, the hydrolysis solution was prepared by dissolving *C. rugosa* lipase into a solution containing buffer, precipitating agent and Gd-HPDO3A in the same concentrations as used previously.

After being removed from the lipidic cubic phase, CP-derivative crystals were soaked for 30 s in native solution containing 30% PEG 400 as a cryoprotectant. In order to eliminate any remaining Gd-HPDO3A complex from the liquor surrounding the derivative crystals, the cryoprotectant solution was free of Gd-HPDO3A complex. The crystals were cooled at 100 K using a cryocooling device from Oxford Cryosystems.

Table 1
Summary of data-collection parameters and of processing statistics.

Values in parentheses refer to the highest resolution shell.

Crystal dimensions (mm)	0.25 × 0.25 × 0.15
Space group	$P4_32_12$
Unit-cell parameters (Å)	a = 77.194, c = 38.438
Resolution range (Å)	40.0–1.71 (1.80–1.71)
No. observed reflections	171753
No. unique reflections	13096
Acentric	10964
Centric	2132
Signal-to-noise ratio $I/\sigma(I)$	6.1 (3.2)
Completeness (%)	99.6 (97.9)
Redundancy	13.1 (11.7)
$R_{\rm sym}$ (%)	7.4 (23.5)
$R_{\rm ano}$ (%)	6.4 (11.2)

2.4. Data collection and data processing

Data were collected with a MAR300 imaging-plate detector using Cu $K\alpha$ radiation from a Rigaku RU-200 rotating-anode X-ray generator equipped with a Supper 7600 double-mirror system. No attempt was made to orient the crystals along any particular crystallographic axis. Using 0.5° steps, data were recorded with a crystal rotation of 180° for high multiplicity. The resolution of data was 1.7 Å, similar to the resolution of the HD-derivative data set (Girard *et al.*, 2002). Intensities were integrated using the program *XDS* (Kabsch, 1988). The integrated intensities were scaled using the program *SCALA* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). A summary of data-collection parameters and processing statistics is given in Table 1.

An anomalous difference Patterson map was calculated using the entire resolution range of data. The resulting w = 1/2 Harker section is shown in Fig. 1. Two gadolinium sites were located at the same positions as the two sites observed in the HD derivative.

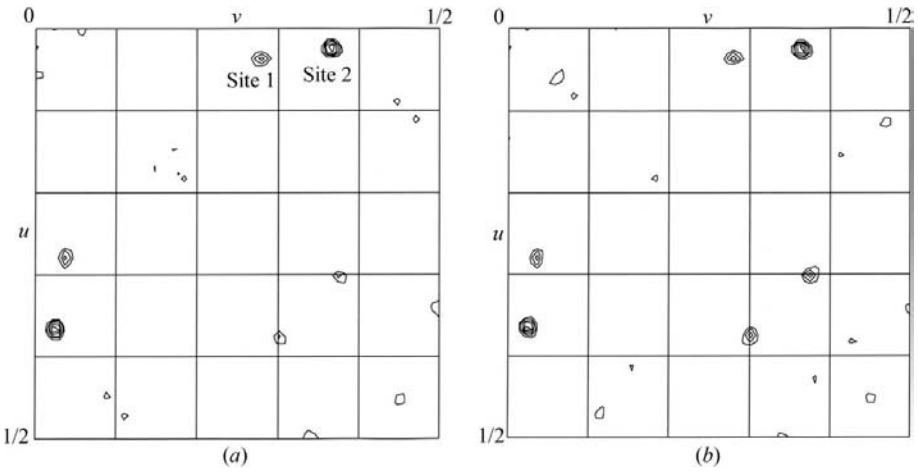

Figure 1
w = 1/2 Harker section of anomalous Patterson maps for (*a*) Gd-HPDO3A CP-derivative crystal and for (*b*) Gd-HPDO3A HD-derivative crystal. Levels are contoured in 1σ steps starting at 2σ.

2.5. Refinement

Using the program *CNS* (Brünger *et al.*, 1998) with the maximum-likelihood target functions, subsequent refinement was performed to evaluate the occupancies of the two gadolinium sites of the CP derivative and to compare them with the refined occupancies of the gadolinium sites of the HD derivative (Girard *et al.*, 2002). Bias in the comparison between $R_{\rm free}$ (Brünger, 1992) was avoided by selecting for the free reflection set of the CP derivative all the reflections of the HD-derivative free set that were present in the CP-derivative data set. The *CNS* scripts were set up to automatically compute a cross-validated σ_A estimate and to evaluate the weight between the X-ray refinement target and the geometric energy function. The entire data set was used without any cutoff. The anomalous signal was taken into account. Corrections for bulk solvent and data anisotropy were applied. The HD-derivative structure (PDB code 1h87) was used in its entirety as the starting model for subsequent refinement of the CP-derivative structure: a rigid-body refinement followed by slow-cooling and individual *B*-factor refinement cycles. The gadolinium and the HPDO3A ligand occupancies were then refined using the *CNS* script *QGROUP*. The final occupancies for each derivative are listed in Table 2.

Table 2
Refined occupancies and thermal *B* factors of the two Gd atoms in the two Gd-HPDO3A lysozyme derivatives.

	100 m*M* Gd-HPDO3A	
	Lipidic cubic phases	Hanging drops
R (%)	19.1	18.0
$R_{\rm free}$ (%)	21.3	21.0
Site 1		
Occupancy	0.77	0.81
B factor (Å^2)	18.8	15.3
Site 2		
Occupancy	0.69	0.73
B factor (Å^2)	19.2	15.9

3. Results and discussion

Studies of the stability of lipidic cubic phases in presence of crystallization screens (Cherezov *et al.*, 2001; Nollert *et al.*, 2002) showed that lipidic cubic phases remain stable when salts or low-molecular-weight PEGs or low concentrations of high-molecular-weight PEGs are added to the solvent. This study also shows that the formation and the stability of the monoolein lipidic cubic phase are not disturbed by Gd-HPDO3A even at high concentration. Therefore, incorporating Gd-HPDO3A into HEWL crystals by co-crystallization in lipidic cubic phase was straightforward.

The unit-cell parameters of the CP-derivative crystals are close to those of the HD-derivative crystals (Girard *et al.*, 2002): a = 77.19, c = 38.44 Å and a = 77.25, c = 38.66 Å, respectively. Binding of the Gd-HPDO3A complex is not prevented by the lipidic cubic phase, as shown by the anomalous Patterson map (Fig. 1). For both types of crystallization methods, the locations and occupancies of the binding sites in the derivative crystals are nearly identical (Table 2), indicating that the binding strength between the Gd-HPDO3A complex and the protein is not affected by the presence of the lipidic matrix, and the refined structures of the two derivatives are isomorphous. Although native HEWL tetragonal crystals were grown in the solvent-channel part of the lipidic cubic phase (Landau *et al.*, 1997), the Gd complex could have been trapped by the lipids. Our experiment shows that this is not the case.

Thus, this experiment shows that the Gd-HPDO3A complex can be a good candidate to obtain heavy-atom derivative crystals grown from lipidic cubic phases. Obtaining good derivative crystals with strong phasing power is often the limiting step in solving new protein structures. Further studies with membrane proteins known to crystallize in lipidic cubic phases are planned in order to test whether CP-derivative crystals with high Gd-site occupancies can be obtained by co-crystallization with Gd-HPDO3A or other Gd complexes.

It has never been possible to prepare lysozyme HD-derivative crystals by soaking native crystals in Gd-HPDO3A, but good gadolinium-derivative crystals of other proteins have been obtained by soaking native crystals in solution containing gadolinium complex (Girard, Stelter, Vicat *et al.*, 2003). Therefore, it will be of great interest to study membrane-protein derivative crystals obtained by soaking native crystals prepared either by the detergent crystallization method or by the lipidic cubic phase crystallization method.

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