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Crystallization and preliminary X-ray diffraction analysis of human phosphate-binding protein

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Human phosphate-binding protein (HPBP) was serendipitously discovered by crystallization and X-ray crystallography. HPBP belongs to a eukaryotic protein family named DING that is systematically absent from the genomic database. This apoprotein of 38 kDa copurifies with the HDL-associated apoprotein paraoxonase (PON1) and binds inorganic phosphate. HPBP is the first identified transporter capable of binding phosphate ions in human plasma. Thus, it may be regarded as a predictor of phosphate-related diseases such as atherosclerosis. In addition, HPBP may be a potential therapeutic protein for the treatment of such diseases. Here, the purification, detergent-exchange protocol and crystallization conditions that led to the discovery of HPBP are reported.

1. Introduction

HPBP was serendipitously discovered from supposedly pure PON1. The structure of HPBP (Morales *et al.*, 2006) relates it to prokaryote phosphate solute-binding protein (SBP; Tam & Saier, 1993; Luecke & Quiocho, 1990; Vyas *et al.*, 2003), which is associated with the ATP-binding cassette transmembrane transporters (ABC transporters; Higgins, 1992). Despite the existence of the ABC transporter in eukaryotes, SBPs have never been described or predicted by genomic databases in eukaryotes.

The complete amino-acid sequence of HPBP (376 amino acids with a predicted molecular weight of 38.4 kDa) was assigned from the electron-density map at the 10% error level (Morales *et al.*, 2006). Surprisingly, the deduced HPBP sequence cannot be retrieved from the human genome or other genomic databases. HPBP is related to a family of eukaryotic proteins that are named DING owing to their four conserved N-terminal residues (Berna *et al.*, 2002). Similarly to HPBP, DING genes are also absent from DNA or RNA databases, although they are likely to be ubiquitous in eukaryotes. This raises numerous questions about the peculiarity of DING genes. The HPBP sequence deduced by crystallography is the first complete sequence of a DING protein and provides a precious basis for understanding the genetic mystery associated with DING proteins.

We have provided evidence that HPBP is a new apoprotein mainly located on HDL (good cholesterol) capable of binding inorganic phosphate ions. Furthermore, HPBP presents 59% amino-acid identity with a protein named crystal-adhesion inhibitor (CAI) that may prevent the development of kidney stones by inhibiting the adhesion of calcium oxalate crystals to renal cells (Kumar *et al.*, 2004). Thus, HPBP could be tentatively regarded as a potential predictor and as a possible therapeutic protein for treatment of phosphate-related disorders, including atherosclerosis.

In this article, we report the purification, detergent-exchange protocol and crystallization conditions that led to the discovery of HPBP.

2. Purification

HPBP was discovered by copurification from an apparently pure PON1 preparation. The HPBP/PON1-containing fractions were obtained according to a protocol based on the method of Gan *et al.*

Table 1
Data collection.

	Native	U peak	U remote
Wavelength (Å)	0.9340	0.722824	0.718758
Energy (eV)	13274.6	17151.5	17249.9
Resolution (Å)	1.89	1.81	2.21
Last resolution shell (Å)	2.01–1.89	1.92–1.81	2.43–2.21
Space group	$C222_1$		
Unit-cell parameters (Å, °)	$a = 97.416$, $b = 87.873$, $c = 90.535$, $\alpha = \beta = \gamma = 90$		

(1991) (Renault *et al.*, in preparation) that was assumed to provide PON1 pure at ≥95%. Briefly, out of date plasma bags from blood donors (Etablissement Français du Sang Rhône-Alpes) were supplemented with $CaCl_2$ to a final concentration of 10 m*M* before the resulting fibrin clot was separated by filtration. The filtrate was then submitted to a pseudo-affinity chromatography on Cibacron Blue 3GA-agarose (type 3000-CL; Sigma) using 50 m*M* Tris–HCl buffer pH 8.0 supplemented with 1 m*M* $CaCl_2$ and 3 *M* NaCl to avoid the adsorption of albumin. Elution of hydrophobic plasma proteins, mainly lipoproteins, was performed using 0.1% sodium deoxycholate and 0.1% Triton X-100 in Tris–HCl buffer. The PON1-containing fractions were pooled and separated from the other HDL-bound proteins, mainly apolipoprotein A-I, by anion-exchange chromatography on DEAE-Sepharose Fast Flow (Pharmacia Biotech) using 25 m*M* Tris buffer containing 0.1% Triton X-100 as starting buffer with a gradient of NaCl (0–0.35 *M*).

3. Crystallization

Pooled HPBP/PON1-containing fractions were dialyzed and concentrated in the presence of C-12 maltoside (0.64 m*M*) using a centrifugation device (Centriprep Amicon, 10 kDa cutoff, Millipore, St Quentin-en-Yvelines, France) to a final absorbance of 2.3 at 280 nm. Light-scattering analysis revealed a homogeneous sample with an apparent molecular weight of about 80 kDa (Josse *et al.*, 2002). This molecular weight was attributed to dimeric PON1 because the existence of HPBP was unknown at this point.

Some dialyzed fractions spontaneously crystallized overnight. Crystal plates were very numerous and very thin (about 1 µm width). Once useless crystals had formed in the absence of precipitant agent, it was impossible to dissolve them again. Thus, crystallization trials were performed quickly after detergent exchange.

Crystallization was performed using the hanging-drop vapour-diffusion method. 3 µl protein solution was mixed with 2 µl of a reservoir solution containing 50 m*M* cacodylate buffer pH 6, 2 *M* ammonium sulfate and 1 m*M* sodium chloride. Small crystals appeared after one week at 277 K. Although the crystals were small (~0.05 × 0.05 × 0.05 mm), they were of good quality (Fig. 1) for diffraction analysis. Until the development of a new purification protocol (Renault *et al.*, 2006), separation by crystallization was the only method to purify PON1 from HPBP.

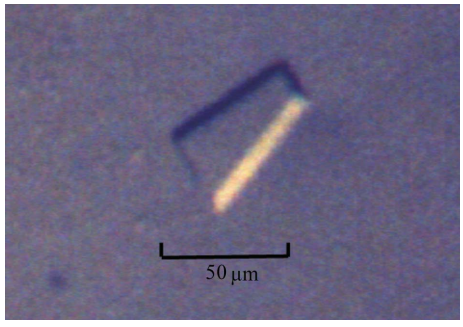

Figure 1
Crystal of HPBP.

4. X-ray diffraction data collection and analysis

X-ray diffraction data were collected at 100 K using synchrotron radiation at the FIP BM30 beamline (ESRF, Grenoble, France) using a 165 mm MAR CCD detector. The crystal was cryoprotected by slowly replacing the drop solution by solution supplemented with 30% glycerol. A native data set was obtained from an initial crystal (Chabrière *et al.*, 2000; Table 1). Heavy-atom derivatives were difficult to obtain. This failure was tentatively explained by the low solvent content ($V_{solvent}$ = 51% for one molecule per asymmetric unit). Thus, in the absence of a derivative, the low-resolution structure (Fig. 2) was solved *ab initio* (*i.e.* with no model or heavy-atom derivative) using a method based on topological criteria (Fokine *et al.*, 2003). Finally, a heavy-atom derivative was obtained by soaking crystals for 3 d in a solution containing a uranium salt at 0.1 m*M* (uranium–plasmocorinth B; Chesne-Seck, 2002; Chesne-Seck *et al.*, 2006). This molecule was specially chosen to produce better uranyl heavy-atom derivatives. Two data sets were collected at the uranium L_{III} edge from one derivative crystal (Table 1). X-ray diffraction data were integrated, scaled and merged with the *XDS*2000 program (Kabsch, 1993) and the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). *CNS* (Brünger *et al.*, 1998) and *SnB* (Weeks & Miller, 1999) were used to find the positions of the three U atoms. The structure was solved by the classical SIRAS method of structure determination at 1.8 Å resolution and will be reported in a paper of biological interest (Morales *et al.*, 2006).

5. Conclusion

Inspection of the resulting electron-density map clearly indicated that the crystallized protein was not PON1. The sequence deduced from the structure was totally unknown and not predicted by the genomic

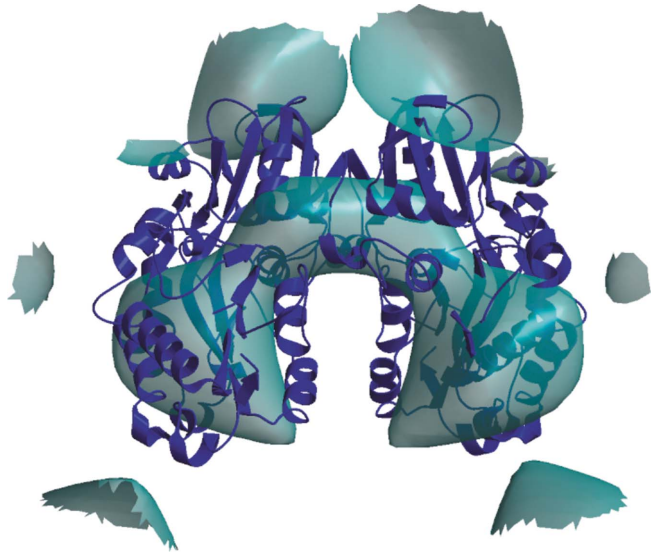

Figure 2
Fourier synthesis calculated at 25 Å resolution with *ab initio* phases obtained by density topological trial and error compared with the ribbon diagram of HPBP.

database. The complete amino-acid sequence was determined from X-ray data. This protein is the first inorganic phosphate transporter characterized in human plasma (Morales *et al.*, 2006). The discovery of this protein by crystallography opens new insight into the physiopathology and medical treatment of phosphate-related diseases such as atherosclerosis.

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