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Purification, crystallization and preliminary X-ray investigation of the complex of human vitamin D binding protein and rabbit muscle actin

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The vitamin D binding protein binds globular actin with high affinity and is involved in the clearance of actin from the blood circulation. A complex of the human vitamin D binding protein and rabbit muscle actin was subjected to purification steps. The pure complex was crystallized using the hanging-drop vapour-diffusion procedure. The best obtained crystals belong to the monoclinic space group $P2_1$, with unit-cell parameters a = 74.44, b = 74.90, c = 88.02 Å, β = 110.19°. A complete data set to 2.4 Å was collected from a single crystal using synchrotron radiation at DESY, Hamburg, Germany.

1. Introduction

Vitamin D binding protein (DBP), also known as Gc-globulin and present in human plasma, is a glycoprotein with a calculated molecular weight of 51 kDa. Its exact size depends on its glycosylation state. Its sequence is homologous to that of albumin, α-fetoprotein and afamin. The nearly identical positioning of the cysteine residues within these proteins predicts a characteristic pattern of disulfide bridges, which indicates that the overall fold of these proteins should be highly homologous (Cooke & Haddad, 1997). For a long time, the binding and transport of vitamin D and its metabolites were considered to be the main functions of DBP. Nowadays, it is clear that DBP is a multifunctional protein (Cooke & Haddad, 1997, White & Cooke, 2000). In addition to the binding of vitamin D and fatty acids and a role in the immune system, DBP has a function in the binding and clearance of actin. DBP binds equimolecular amounts of actin with high affinity ($K_d = 10^{-9}$ M; Van Baelen *et al.*, 1980). The binding is non-covalent and does not interfere with the binding of vitamin D ligands. According to biochemical studies, the actin-binding domain is located between residues 350 and 403 at the carboxy terminus of the DBP molecule (Haddad *et al.*, 1992).

Actin is the most abundant protein present in human cells. It has a molecular weight of 42 kDa (Lind *et al.*, 1986). The DBP-binding site is localized in the carboxy-terminal half of the actin molecule between residues 360 and 372 in subdomain I (Houmeida *et al.*, 1992). DBP appears to share its binding site on the actin molecule with profilin, but at a 1000-fold greater affinity. In contrast, the actin-binding site for DNase I, involving subdomains II and IV of actin, is distinct from that for DBP and these proteins can form a equimolar triprotein complex (Van Baelen *et al.*, 1980; Kabsch *et al.*, 1990). DNase I does not alter the actin-binding affinity of DBP (McLeod *et al.*, 1989). Actin is involved in muscle contraction, cell motility, cellular excretion, mitosis and the construction of the cytoskeleton (Reisler, 1993). Actin can exist as monomers (G-actin) or can polymerize into long filaments (F-actin). *In vivo*, an equilibrium between G- and F-actin is achieved owing to the action of many different actin-binding proteins. *In vitro*, under physiological buffer conditions, G-actin polymerizes spontaneously into filaments (Steinmetz *et al.*, 1997).

Actin can be released in the serum during conditions involving severe cell injury such as trauma, shock, sepsis and fulminant hepatic necrosis (Lee *et al.*, 1985; Haddad *et al.*, 1990). To clear actin from the circulation, DBP and plasma gelsolin cooperate in the important extracellular actin-scavenger system. Whereas plasma gelsolin binds to F-actin and severs it into monomers, DBP binds and sequesters these monomers, thus preventing the formation of long actin filaments in the bloodstream. The clearance of actin from the circulation consists of two phases: a rapid Ca^{2+}-dependent phase arising from the rapid severing of F-actin by gelsolin and a slow Ca^{2+}-independent phase arising from the action of DBP, to which the actin monomers bind preferentially. Both phases are saturable (Janmey & Lind, 1987). The DBP–actin clearance process, however, is much faster than the clearance of free DBP (Goldschmidt-Clermont *et al.*, 1988; Lee & Galbraith, 1992).

The presence of actin filaments in the blood circulation is dangerous and could be fatal (Lee & Galbraith, 1992). The viscosity of the blood could increase and actin interferes in the clot formation by its ability to aggregate platelets (Lind *et al.*, 1986, Vasconcellos &

Lind, 1993). *In vivo* saturation of the plasma scavenger system for actin induces intravascular filament formation, microthrombi and endothelial injury, especially in the pulmonary circulation (Haddad *et al.*, 1990; Erukhimov *et al.*, 2000). In patients with multiple trauma, a marked consumption of DBP appears immediately after the trauma. A lack of DBP is related to non-survival and the severity of the trauma (Dahl *et al.*, 1998). Also, in patients with fulminant hepatic failure, measurement of DBP levels correctly predicted all patients dying of hepatic failure. Measurement of DBP levels might be a useful test for patients being considered for transplantation (Lee *et al.*, 1995).

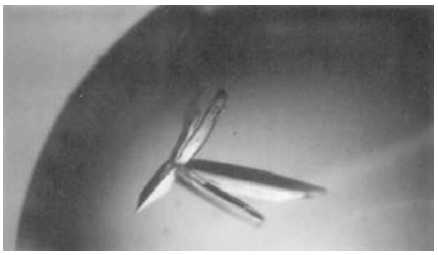

Figure 1
Typical DBP–actin crystals. The dimensions of the largest crystal were 0.5 × 0.15 × 0.1 mm.

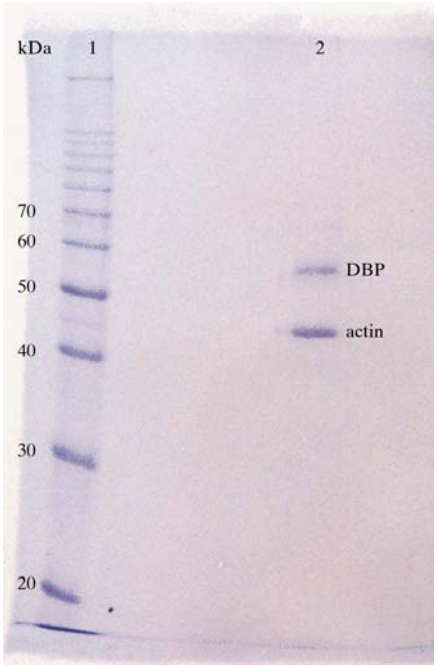

Figure 2
12% SDS–PAGE analysis of the DBP–actin crystals. Lane 1 shows the molecular mass markers. The 10 kDa Protein Ladder, Gibco BRL (Life Technologies, Merelbeke, Belgium) was used. Lane 2 shows DBP and actin from the dissolved DBP–actin crystals. Proteins were visualized by Coomassie Blue staining.

To obtain a better insight into the nature of the interaction between the DBP and actin molecules, we have initiated a crystallographic study of the complex.

2. Materials and methods

2.1. Purification

DBP was isolated from pooled human serum as described previously (Verboven *et al.*, 1995). Rabbit muscle actin was obtained from Sigma. A stock solution of actin (2 mg ml^{-1}) was prepared in G-buffer (2 m*M* Tris–HCl pH 7.6, 0.2 m*M* ATP, 0.5 m*M* mercaptoethanol and 0.2 m*M* $CaCl_2$) and kept on ice at all times. Prior to the preparation of the actin–DBP complex, the depolymerization of actin was further promoted by an additional 20-fold dilution in G-buffer. Finally, the actin–DBP complex was prepared by incubating the diluted actin with equimolar concentrations of DBP for 10 min at room temperature. Subsequently, the actin–DBP complex was separated from free DBP and from free actin by gel filtration on a custom-made Superdex 200 HR 16/50 column (dimensions, 1.6 × 50 cm; particle size, 13 µm; manufacturer, Pharmacia Biotech, Uppsala, Sweden) previously equilibrated in 100 m*M* Tris–HCl pH 7.6, 0.2 m*M* ATP and 0.5 m*M* mercaptoethanol.

2.2. Crystallization and data collection

The protein solution was further concentrated using ultrafiltration with Microcon devices (Amicon, USA) to a concentration of 10 mg ml^{-1}. Crystallization was performed in VDX plates (Hampton Research, USA) by the hanging-drop vapour-diffusion method at a temperature of 277 K. The first crystallization trials were performed using the screening method of Jancarik & Kim (1991). Conditions favouring crystal growth were optimized using different buffers, pH, precipitants, protein concentration, drop size and additives.

To identify the content of the obtained crystals, some of the crystals were dissolved and subjected to SDS–PAGE. The crystals were washed several times in their crystallization solution and further dissolved in 6 µl SDS sample buffer. This solution was boiled for 5 min and analyzed by 12% SDS–PAGE according to the method of Laemmli (1970).

Diffraction data were collected at the EMBL BW7B beamline at the Doris storage ring, DESY, Hamburg with a MAR 345 image plate. The experiments were performed at 100 K using an N_2 gas stream to cool the crystals (Oxford cryogenic cooling system). The data were collected at a wavelength of 0.8423 Å. The crystal-to-detector distance was 450 mm. The crystal rotation angle per image was 1.0°. All data were processed and scaled with the *HKL* package (Otwinowski & Minor, 1997). The self-rotation function was generated using the *CNS* program package (Brunger *et al.*, 1998).

Table 1
Data-collection statistics.

Values in parentheses are for the outer resolution shell, 2.44–2.40 Å.

Resolution range (Å)	20–2.40
No. of reflections measured	97747
No. of unique reflections	33252
Redundancy	2.9 (2.6)
Completeness (%)	93.3 (93.9)
$R_{\rm merge}$† (%)	4.2 (25.0)
$R_{\rm meas}$‡ (%)§	5.1 (30.9)
$\langle I/\sigma(I)\rangle$§	12.2 (1.8)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$. ‡ $R_{\rm meas} = \sum_{hkl}[n_{hkl}/(n_{hkl}-1)]^{1/2}\sum_i^{n_{hkl}} |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i^{n_{hkl}} I_i(hkl)$. § These values were obtained with the program *NOVEL_R* (Diederichs & Karplus, 1997*a*,*b*).

3. Results and discussion

The best crystals were grown from 13% polyethylene glycol 8000, 0.2 *M* magnesium acetate, 0.1 *M* sodium cacodylate buffer pH 6.3 and 20% glycerol. This solution was suitable for cryoprotection of the crystals. Drops consisting of 3 µl reservoir solution and 3 µl protein solution were equilibrated against 750 µl reservoir solution. Crystals, as shown in Fig. 1, appeared after approximately two weeks. To verify whether DBP and actin were present in these crystals, some crystals were dissolved and analyzed by SDS–PAGE. The result is shown in Fig. 2. The two bands which can be clearly recognized in lane 2 correspond to DBP (the 55 kDa band) and actin (the 43 kDa band). The crystals diffract to at least 2.4 Å (Fig. 3). A complete data set to this resolution was collected from such a crystal. The results of the data processing are summarized in Table 1. The unit-cell parameters are $a = 74.44$, $b = 74.90$, $c = 88.02$ Å, $\beta = 110.19°$. The diffraction pattern shows a symmetry consistent with the monoclinic space groups $P2$ or $P2_1$, showing two related asymmetric units in the unit cell. The systematic absence of $k = 2n + 1$ reflections along the $0k0$ axis suggests that the space group is $P2_1$. In order to estimate the number of molecules in the asymmetric unit, the Matthews coefficient ($V_{\rm M}$) was calculated (Matthews, 1968). According to the unit-cell parameters, the calculated volume of the unit cell is

46.06 × 10^4 Å^3. Using a total molecular weight of 93 300 Da and two molecules in the unit cell, the Matthews coefficient (V_M) becomes 2.47 Å^3 Da^{-1}, which is in the range typical for protein crystals. It is therefore assumed that there is only one DBP–actin molecule in the asymmetric unit. The absence of a significant peak in the self-rotation function supported this assumption. The fractional volume occupied by the solvent (V_{solv}) is calculated to be 0.50 (Matthews, 1968).

The collected data set made it feasible to find a molecular-replacement solution using the X-ray structures of actin (Kabsch *et al.*, 1990) and DBP (Verboven, 1998). Of particular interest are possible conformational changes in the DBP molecule arising from the binding to actin. Indeed, interaction with actin alters certain physicochemical properties of DBP. The hydrophobicity of DBP alters by complexation with actin; isoelectric focusing revealed that the DBP–actin complex is more acidic than either protein alone (Goldschmidt-Clermont *et al.*, 1987). The identification of the binding site of both proteins may also reveal why DBP is the only member of the albumin family that is able to bind actin. Because conditions favouring crystallization of G-actin also induce its polymerization to F-actin, it has been difficult to obtain suitable G-actin crystals for X-ray investigation. To overcome this problem, the structure of actin complexed to actin-binding proteins such as DNase I and gelsolin segment 1 has been solved (Kabsch *et al.*, 1990; McLaughlin *et al.*, 1993). Solving the DBP–actin structure will also allow the comparison of the actin conformation with its conformation in the already known complexes. Importantly, knowledge of the DBP–actin structure will contribute to the understanding of the way DBP prevents the polymerization of actin.

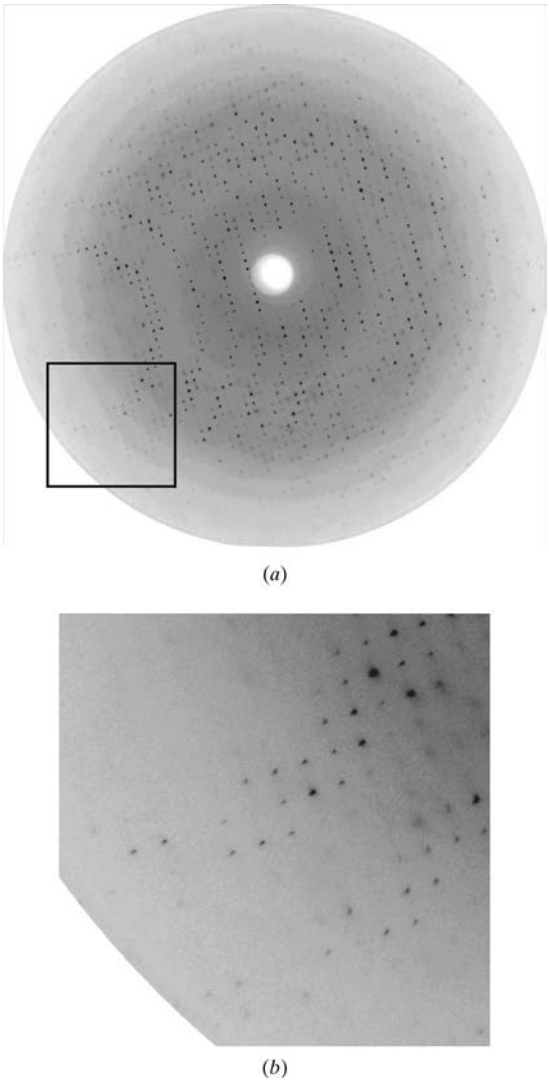

Figure 3
(*a*) Typical diffraction image of the DBP–actin crystal. The resolution of the outer edge of the image is 2.3 Å. (*b*) An enlarged image of the indicated area in (*a*).

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