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Rapid Communication

Crystallization and X-ray Investigation of Vitamin D-binding Protein from Human Serum. Identification of the Crystal Content

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Vitamin D-binding protein (DBP), a multifunctional, highly polymorphic glycoprotein responsible for the transport of vitamin D and for sequestering extracellular actin, was isolated from human serum and crystallized using vapour diffusion methods. The crystals were grown from 7.5% v/v polyethylene glycol 400 and 0.1 M acetate buffer at pH 4.6. These crystals show diffraction patterns consistent with the tetragonal space groups $P4_1$ and $P4_3$ with unit cell dimensions $a = b = 135.5(4)$ Å and $c = 75.9(4)$ Å. They diffract to 2.3 Å. Using polyacrylamide gel electrophoresis it was shown that according to their electrophoretic mobility the O-glycosylated isoforms, with a terminal sialic acid residue, are absent in the crystals.

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INTRODUCTION

Vitamin D-binding protein (DBP), also known as Group-specific component or Gc-globulin, is a 51 kDa glycoprotein present in human serum. Its sequence is homologous to that of serum albumin and α-fetoprotein [1] and to that of afamin [2]. Moreover the tertiary structure of the proteins is expected to be similar [1].

DBP is a highly polymorphic protein. Although there are more than 120 known alleles which encode for human DBP [3], the three most common ones are Gc1F, Gc1S and Gc2. Whereas the Gc2 allele encodes for a single isoform, each of the Gc1 alleles produces two isoforms, Gc1c and O-glycosylated Gc1a with a terminal sialic acid residue. Therefore DBP prepared from pooled serum contains at least 5 major DBP isoforms.

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Over the past couple of decades it has become clear that DBP is a multifunctional protein. DBP is the major plasma carrier of vitamin D_3 and of all its metabolites such as 25-hydroxyvitamin D_3 (25OHD) and 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D$) [4]. The active vitamin D hormone, $1,25(OH)_2D$, is one of the key hormones for calcium and bone homeostasis, but also has a function in the regulation of cellular differentiation and proliferation [5]. The high affinity in combination with the high concentration of DBP markedly decreases the free concentration of $1,25(OH)_2D$. It, therefore, limits the bioavailability of the vitamin D metabolites but prolongs their halflife. Synthetic analogs with increased potency on cellular differentiation but low calcemic activity all have very low DBP binding affinity indicating that the transport function is more than a mere physical interaction [6].

In vitro DBP is capable of binding monomeric actin and slowly depolymerizes filamentous actin [7]. In cooperation with actin-severing gelsolin, DBP may play a crucial role *in vivo* in the important actin

scavenger system. Whereas gelsolin severs actin filaments, the generated globular actin is sequestered by DBP thereby preventing spontaneous formation of filaments [8].

There are several indications for a role of DBP in the immune response. DBP associates with membrane immunoglobulins and immunoglobulin G [9]. DBP enhances the neutrophil chemotactic activity of complement derived peptides, C5a and C5a des Arg [10, 11]. Moreover DBP was also found to be a precursor of a macrophage activating factor necessary for osteoclast activation [12, 13].

To obtain a better insight in these different interactions, we have undertaken a crystallographic study of DBP.

MATERIALS AND METHODS

DBP was either isolated from serum of an individual with the Gc2-2 phenotype (one isoform) or from pooled human serum (five isoforms) by means of affinity chromatography on immobilized anti-DBP antibodies. The protein solution was further purified using ion exchange chromatography (HiLoad 16/10, Q Sepharose HP). The column was eluted with a NaCl gradient containing 25 mM Tris–HCl buffer, pH 7.4. The protein was concentrated using ultrafiltration with Microcon devices (Amicon, Beverly, U.S.A.).

These two protein preparations were used in vapour diffusion crystallization experiments in Linbro multiwell tissue culture plates (ICN Biomedicals, Costa Mesa, U.S.A.). Both salt solutions and polyethylene glycol (PEG) solutions were tested as precipitants [14]. Other variables were the presence of added 25OHD, temperature, pH, protein and precipitant concentration. The crystal parameters were determined with a X-1000 multiwire area detector (Siemens, U.S.A.). The X-ray source was a sealed tube with a copper anode producing CuKα-radiation. The source was operated at 45 kV and 35 mA using a 0.5 mm collimator. The Ficoll method was used to measure the crystal density [15].

To identify the content of the crystals, several crystals were separated from their mother liquor, dried on filter paper, washed several times with pure water, redissolved in a buffer solution of 20 mM Tris–HCl, pH 7.5, and finally analyzed by polyacrylamide gel electrophoresis. A 4.5% stacking gel and a 10% resolving gel was used. Approximately 1 μg of protein was applied. The electrophoresis was performed at pH 8.6 for 5 h. After fixation of the proteins, the gel was silver stained.

RESULTS AND DISCUSSION

Two DBP preparations, one isolated from serum of an individual with the Gc2-2 phenotype (one isoform) and the other isolated from pooled human serum (five isoforms), were submitted to crystallization trials. Crystals were obtained in acetate buffer solutions containing PEG 400 or PEG 4000. Although the two DBP preparations produce crystals, only those from "pooled" DBP could be grown without streak seeding. The best crystals were obtained from a 7.5% v/v PEG

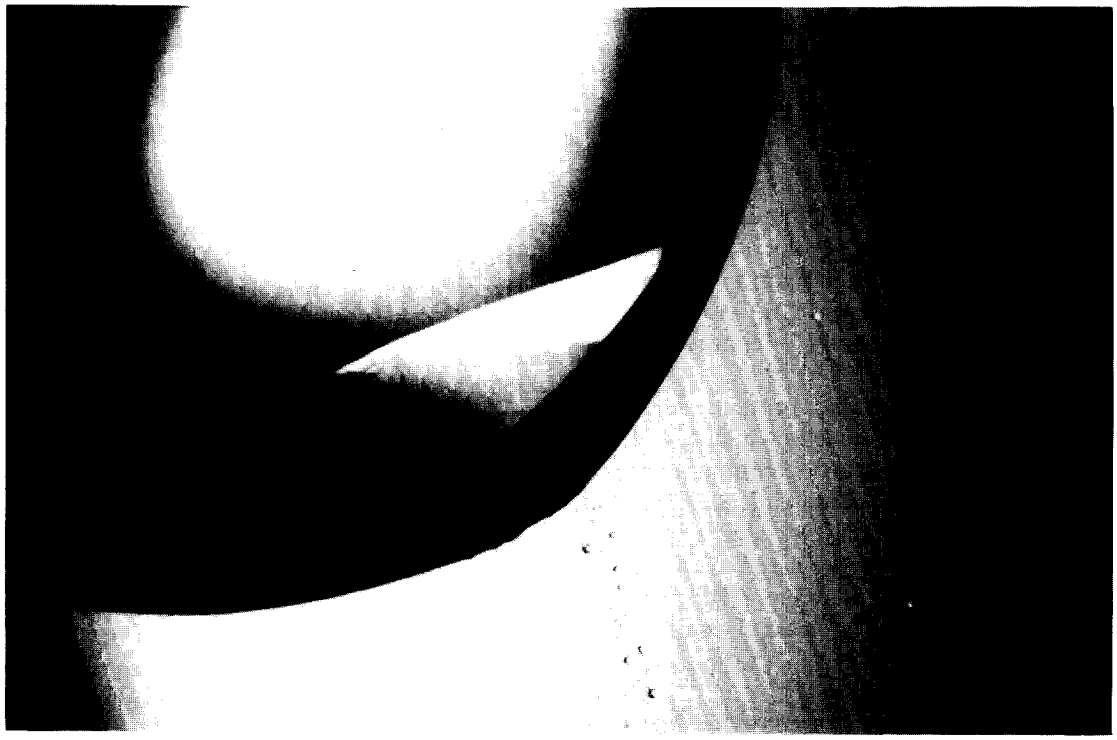

Fig. 1. DBP crystal (0.7 × 0.4 × 0.2 mm).

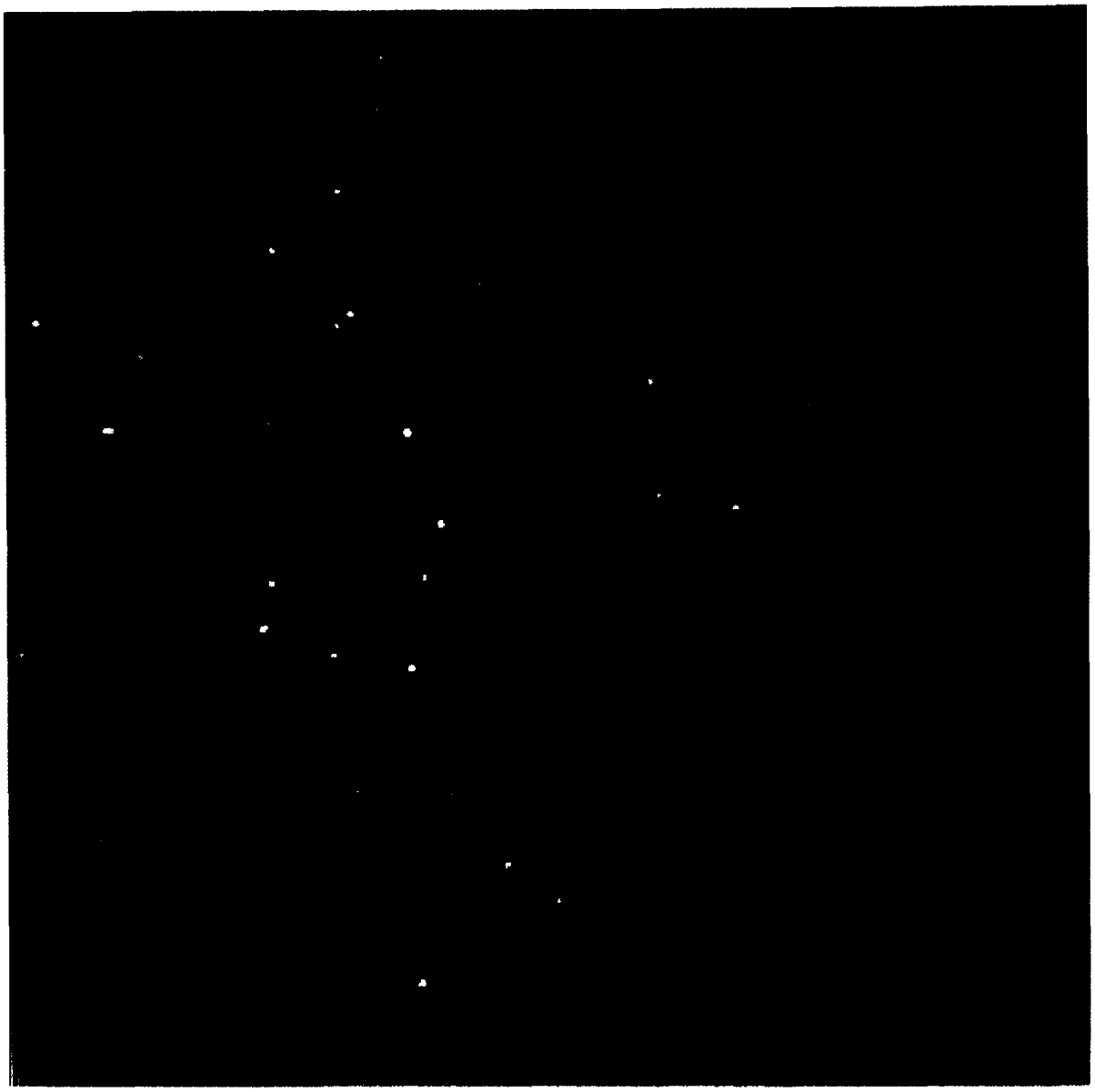

Fig. 2. Diffraction image of DBP crystal taken with a crystal to detector distance of 130 mm and an oscillation of the crystal of 0.2°.

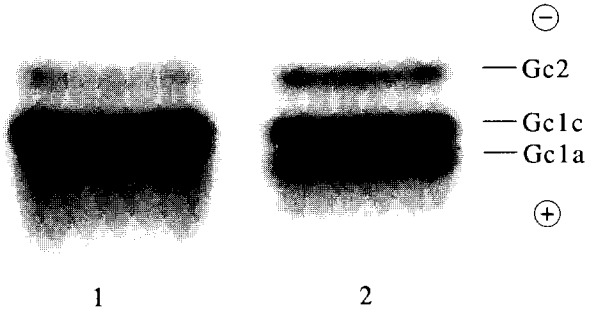

Fig. 3. Polyacrylamide gel electrophoresis of crystallized DBP (lane 1) and pure "pooled" DBP (lane 2).

400 solution containing 0.1 M acetate buffer, pH 4.6, without addition of 25OHD, using the hanging drop technique at room temperature (Fig. 1). The drops consisted of 3 μl precipitant solution and 3 μl protein solution with a protein concentration of 30 mg/ml. The crystals appeared after 1 week. Crystals as the one shown in Fig. 1 diffract up to 2.3 Å (Fig. 2). The unit cell dimensions are $a = b = 135.5(4)$ Å and $c = 75.9(4)$ Å, $\alpha = \beta = \gamma = 90°$. The diffraction pattern shows a 4/m symmetry and systematic absences for reflections $l \neq 4n$ which is consistent with the tetragonal space groups $P4_1$ or $P4_3$. The measured crystal density of 1.06(1) g/ml indicates that there is only one DBP molecule per asymmetric unit. This corresponds to a very high crystal solvent content of about 80%.

In comparison with previously reported preliminary crystallographic studies on DBP [16, 17] our crystals were grown using a different purification procedure for DBP and different crystallization conditions. Our crystals show notably better diffraction than those grown by Vogelaar *et al.* [17] (2.3 Å compared to 3.4 Å resolution) who estimated the same solvent content of the crystal. Koszelak *et al.* [16] reported monoclinic crystals of DBP grown in the presence of 25OHD.

Since the best crystals were obtained from "pooled" DBP it was of interest to know which isoforms crystallized. Therefore some of the crystals were redissolved and analyzed by polyacrylamide gel electrophoresis (Fig. 3). The results indicate that the isoforms with highest mobility (those containing a terminal sialic acid residue) are not present in our crystals. Only the non-glycosylated Gc1 isoforms (Gc1c) and the Gc2 isoform are incorporated in the crystals. But, according to the staining intensities of the different protein bands it is also clear that the Gc1c isoforms show a better tendency to be incorporated in the crystals than the Gc2 isoform.

These results confirm that the number of isoforms in the protein solution should be as small as possible. On an experimental basis we can exclude the Gc2 single isoform sample. Therefore we decided to isolate DBP from a homozygous carrier of the Gc1S (or Gc1F) allele. With the new Gc1S DBP preparation we hope to obtain even better quality crystals and higher resolution data. Our current data set is, however, largely suitable for the analysis of the three dimensional structure and is being used for structure determination applying the molecular replacement strategy.

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