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Cross-talk among structural domains of human DBP upon binding 25-hydroxyvitamin D

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Abstract

Serum vitamin D-binding protein (DBP) is structurally very similar to serum albumin (ALB); both have three distinct structural domains and high cysteine-content. Yet, functionally they are very different. DBP possesses high affinity for vitamin D metabolites and G-actin, but ALB does not. It has been suggested that there may be cross-talk among the domains so that binding of one ligand may influence the binding of others. In this study we have employed 2-p-toluidinyl-6-sulphonate (TNS), a reporter molecule that fluoresces upon binding to hydrophobic pockets of DBP. We observed that recombinant domain III possesses strong binding for TNS, which is not influenced by 25-hydroxyvitamin D₃ (25-OH-D₃), yet TNS-fluorescence of the whole protein is quenched by 25-OH-D₃. These results provide a direct evidence of cross-talk among the structural domains of DBP.

Keywords

Structural domains of vitamin D binding protein (DBP); 25-hydroxyvitamin D₃ (25-OH-D₃); 2-p-toluidinyl-6-sulphonate (TNS): reporter molecule; conformational change; cross-talk among domains

Introduction

Vitamin D-binding protein (DBP) or group specific component (Gc) is a relatively abundant, polymorphic and sparsely glycosylated serum protein with multiple functions. DBP binds vitamin D and its metabolites with high affinity ($K_d = 10^8\text{--}10^{11}\text{M}^{-1}$); and this property (of DBP) is manifested in the organ-specific transportation of vitamin D and its metabolites to target tissues and stepwise oxidation of vitamin D₃ into its physiologically active metabolite, 1 α ,25-dihydroxyvitamin D₃ [1–3]. DBP also binds serum G-actin with high affinity ($K_d \cong 10^8\text{M}^{-1}$). Such an interaction is aided by plasma gelsolin, and prevents G-actin from polymerizing into F-actin and blocking arteries under conditions of cellular injury or death. This property has serious implications in thrombosis and heart attack [4–8]. DBP also binds chemotactic agents such as C5a and C5a des Arg, thus enhancing complement activation on neutrophil chemotaxis [9,10]. In addition, DBP binds saturated and poly-unsaturated fatty acids with high affinities [11,12]. Moreover, a post-translationally modified version of DBP (DBP-

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macrophage activating factor) has been shown to have strong macrophage- and osteoclast-activating [13–17], as well as antiangiogenic and anti-tumor properties [18,19].

DBP belongs to the albumin gene-family; and it is structurally highly homologous with albumin (ALB), alpha-feto protein and afamin [20–22]. All these proteins are characterized by modular structures with three structural domains (domains I–III) and high cysteine (Cys)-content. In the case of DBP domain III is considerably truncated compared with other members of this gene-family. In addition, all the Cys residues in DBP, in contrast with ALB, are oxidized to disulfide linkages.

During the past decade several structure-function studies were carried out to strongly suggest that different domains of DBP are responsible for its various ligand-binding activities. For example, domain I was shown to be exclusively reserved for vitamin D sterol-binding [23–27], while G-actin-binding takes place in domain III [23,26,27]. DBP-*maf* activities, which are manifested by the partial deglycosylation of carbohydrate-containing DBP, are also restricted to domain III of the protein [28]. In the light of these observations it has been suggested that there may be a cross-talk among the structural domains of DBP so that binding of one ligand may influence the binding of another.

In this investigation we probed 25-hydroxyvitamin D₃ (25-OH-D₃)-binding (the strongest binder among all naturally occurring vitamin D metabolites) by human DBP using 2-p-toluidinyl-6-sulphonate (TNS) as a fluorescent reporter molecule. Results of the above studies are discussed in this communication in the light of multiple ligand-binding by DBP and its probable physiological implications.

Materials and methods

Purified human DBP was obtained from commercially available pooled human serum (American Red Cross, Dedham, MA) by a ligand affinity chromatographic method developed in our laboratory [29]. The recombinant C-terminal domain of hDBP (domain III, hDBP 277–458) was expressed in bacteria by our published procedure [25,30]. All other chemicals and biochemicals were obtained from Sigma-Aldrich Chemical Co., Milwaukee, WI, except [26(27)-³H]25-hydroxyvitamin D₃ (³H-25-OH-D₃, specific activity 20 Ci/mmol), that was purchased from DuPont – NEN, Boston, MA.

hDBP + TNS + 25-OH-D₃ (various amounts)

20 µg samples of hDBP in TRIS buffer, pH 8.4 was incubated with TNS (2 µg) at 25°C for 20 min. After this period, different amounts of 25-OH-D₃ (0.001, 0.01, 0.1, 1 µg) were added to the DBP-TNS solutions, and fluorescence intensities were recorded in a Hitachi F 2000 Fluorescence Spectrophotometer. In a separate experiment, fluorescence spectra of TNS alone (in TRIS buffer), TNS + 25-OH-D₃, hDBP + TNS, and hDBP + TNS + 25-OH-D₃ (1 µg) were recorded.

hDBP + ³H-25-OH-D₃ + TNS (various amounts)

Samples of hDBP (20 µg each) in TRIS buffer, pH 8.4 were incubated for 20 hours at 4°C with ³H-25-OH-D₃ (4,000 cpm) either without or with various amounts of TNS (0.25 – 8.5 µg, as shown in Figure 2). After the incubation, the samples were incubated on ice with Dextran-coated charcoal for 15 min. The samples were centrifuged (5,000 rpm, 4°C). Supernatant from each sample was mixed with scintillation cocktail and counted for radioactivity.

Competitive binding assay of hDBP with ^3H -25-OH- D_3 and a fixed amount of TNS

Samples (20 μg) of hDBP were incubated at 4°C for 20 hours with ^3H -25-OH- D_3 (4,000 cpm) either with or without TNS (1.5 μg) and an increasing concentration of 25-OH- D_3 (0.05 – 51.2 ng, as shown in Figure 3). Another set of sample without any TNS was treated the same way (control). The rest of the procedure is same as described earlier.

hDBP 277-458 + TNS + 25-OH- D_3

Samples of hDBP 277-458 (20 μg each) in TRIS buffer, pH 8.4 were incubated with TNS (2 μg) at 25°C for 20 min. After this period, 25-OH- D_3 (1 μg) was added to the solutions, and fluorescence spectra were recorded.

Results and discussion

DBP, similar to other members of albumin (ALB) gene-family has a triple domain modular structure and a large number of cysteine (Cys) residues. All twenty eight (28) Cys residues in DBP are engaged in forming fourteen (14) disulfide bonds leading to the formation of these domains. Domain I spans about 200 amino acids and stabilized by 5 disulfide bonds, and contains the only Trp (145) residue which is involved in vitamin D sterol-binding [31]. Domain II is about one hundred and seventy five (175) amino acids long and contains six (6) disulfide bonds. Domain III spans about eighty five (85) amino acid residues (starting from amino acid residue 375) to the carboxy terminus and is stabilized by two (2) disulfide bonds.

ALB also has a triple-domain structure like DBP, but, in spite of high sequence and structural homology, DBP and ALB are functionally quite different. For example, DBP is a highly specific binder of vitamin D sterols and G-actin, while ALB is not. Moreover, DBP-*maf*-like activities of ALB are unknown to date. Accommodation of multiple high-specificity binders and multifunctional nature of DBP raises the possibility that binding of one ligand might influence the binding of other(s) via 'cross-talk' among interacting domains, and such a process ultimately influence its functions. However, to date there has not been any direct evidence of such cross-talk among domains of DBP.

Changes in the intrinsic fluorescence of proteins (of aromatic amino acid residues) upon ligand/substrate- binding have been used quite extensively to study the micro-environment around these amino acids [32]. In addition, certain fluorescent hydrophobic molecules have been used as reporter molecules to probe micro-environment in proteins. 2-p-Toluidinylnaphthalene-6-sulfonate (TNS) is such a molecule. TNS does not fluoresce in an aqueous solvent, but fluoresces strongly in non-polar organic solvents and when bound to hydrophobic regions of a protein. In some cases this binding is strongly influenced by the binding of the natural ligand/ligands. For example, TNS produces high quantum-yield fluorescence with serum ALB, beta-lactalbumin and chymotrypsin, while with other proteins, like lysozyme, IgG and ovalbumin quantum-yields are considerably lower [32].

Goldschmidt-Clermont *et al.* showed that DBP displays strong fluorescence upon binding TNS, and this fluorescence is reduced in a dose-dependent manner by G-actin, and fluorescence is completely quenched at a concentration ratio of 1:1 [33]. Dose-dependent decrease in TNS-fluorescence was explained as a representation of a change in conformation of DBP upon binding G actin, instead of a simple displacement of TNS by G-actin. Such alteration in physicochemical properties has been reported in the literature. For example, binding between hemoglobin and haptoglobin has been shown to be accompanied by altered hydrophobicity and

anodal shift in isoelectric focusing [34]. We carried out the present study to investigate the effect of 25-OH-D₃ on TNS-binding by hDBP.

Effect of 25-OH-D₃ on DBP-TNS fluorescence

We observed that TNS-fluorescence decreased steadily with increasing concentration of 25-OH-D₃, and fluorescence intensity was almost completely obliterated by 1 µg of 25-OH-D₃ (Figure 1). In support of this observation the strong hDBP-TNS fluorescence peak at 435 nm (Figure 1, Inset, Curve A) was almost completely obliterated by 25-OH-D₃ (1 µg) (Figure 1, Inset, **Curve B**), while a combination of TNS and 25-OH-D₃ (1 µg) had very little fluorescence (Figure 1, Inset, **Curve C**).

This dose-dependent decrease in TNS-fluorescence by 25-OH-D₃ can be explained by either a direct competition between TNS and 25-OH-D₃ for binding site on DBP, or a change in physicochemical property (hydrophobicity, conformation) of DBP upon 25-OH-D₃-binding so that hydrophobic TNS-binding site/sites become progressively less available upon addition of 25-OH-D₃. To determine the mechanism of the above observation we carried out the following experiment.

Binding assay of hDBP with ³H-25-OH-D₃ in the presence of various amounts of TNS

Results of this assay show that ³H-25-OH-D₃-binding by DBP is not influenced at all by TNS (Figure 2), strongly suggesting that there is no direct competition between 25-OH-D₃ and TNS for binding site/sites in hDBP. However, it could not be ascertained (from these results) whether nature of 25-OH-D₃-binding to DBP (binding affinity) is altered by the binding of TNS. This was determined by the following experiment.

Assay to determine whether binding affinity of hDBP for 25-OH-D₃ is altered by TNS-binding

The competitive binding assay curves of DBP and ³H-25-OH-D₃ in the presence of 15 µg of TNS or in its absence are almost overlapping, indicating that TNS does not significantly alter interaction between 25-OH-D₃ and DBP qualitatively and quantitatively (Figure 3).

Collectively the above results re-emphasize that there is no direct competition between 25-OH-D₃ and TNS for DBP-binding. In addition these studies indicate that TNS-binding does not alter the nature of binding between 25-OH-D₃ and DBP.

In the past our laboratory and others have shown that vitamin D sterol binding by DBP is largely restricted to domain I of the protein [23–25], while G-actin-binding takes place largely via domain III of DBP [23]. Furthermore, DBP-TNS fluorescence is reduced in a dose-dependent manner by G-actin, and is completely quenched at a concentration ratio of 1:1 [33], suggesting that TNS-binding might take place largely in domain III of DBP. In order to investigate that possibility we carried out TNS-binding by a recombinant domain III (mostly domain III and a small segment of domain II) of hDBP in the presence and in the absence of 25-OH-D₃.

Fluorescence spectra of recombinant hDBP 277-458 with TNS and 25-OH-D₃

We observed that hDBP 277-458 alone does not have any significant fluorescence activity (Figure 4, **Curve B**), but it displays strong fluorescence with a maximum at 435 nm in the presence of TNS (Figure 4, **Curve A**, please note the change in the scale of the Y-axis from

Figure 1, Inset). This peak was not at all influenced by the addition of an excess of 25-OH-D₃ (Figure 4, **Curve C**).

Collectively the above results suggest that major TNS-binding pocket in DBP may lie in domain III (C-terminal) of the protein. It could be argued that since domain III is not involved in 25-OH-D₃-binding [23–26], TNS-fluorescence by this recombinant domain III is not influenced by 25-OH-D₃-treatment. However, this is in contrast with results displayed in Figure 1 where we demonstrated that TNS-fluorescence by full-length DBP is almost completely quenched by 25-OH-D₃. This apparent anomaly can be explained by significant conformational change in the whole protein upon 25-OH-D₃-binding (in domain I) to influence TNS-binding in domain III. As a result TNS-fluorescence of full-length hDBP is quenched by 25-OH-D₃ in a dose-dependent manner (Figure 1).

In summary, results of this study strongly implies that a considerable conformational change takes place in hDBP molecule upon binding 25-OH-D₃ in domain I of the protein; and this change is propagated into domain III to strongly influence TNS-binding in domain III of the protein, suggesting a cross talk among the domains. DBP is a multi-functional protein. Therefore, this direct evidence of cross talk has strong implications in the structure-functional aspects of this serum protein.

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Figure 1.

TNS-fluorescence assays of human serum DBP (hDBP) in the presence of various amounts of 25-OH-D₃. Inset: TNS- fluorescence spectra of hDBP + TNS (Curve A), hDBP + TNS + 25-OH-D₃ (1 μ g) (Curve B), and TNS + 25-OH-D₃ (Curve C).

Figure 2.
 ^3H -25-OH- D_3 binding assay of hDBP in presence of various amounts of TNS.

Figure 3. Competitive binding assay of hDBP and ^3H -25-OH-D₃ in the presence or in the absence of 15 μg of TNS.

Figure 4. TNS-fluorescence spectra of hDBP 277-458 peptide + TNS (Curve A), hDBP 277-458 peptide alone (Curve B), and hDBP 277-458 peptide + TNS + 25-OH-D₃ (Curve C).