Roles of the Structure and Orientation of Ligands and Ligand Mimics inside the Ligand-Binding Pocket of the Vitamin D-Binding Protein†

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ABSTRACT: 1α,25-Dihydroxyvitamin D_3, the vitamin D hormone, manifests its diverse biological properties by specifically binding to the vitamin D sterol-binding pockets of vitamin D-binding protein (DBP) and vitamin D receptor. In the past, several affinity, photoaffinity, and chemical modification studies have been carried out to probe the vitamin D sterol-binding pocket of DBP and to evaluate the relationship between the structure of this pocket and the functions of the protein. In the present study, we examined the steric requirements inside this pocket by considering conformational structures of various bromoacetate derivatives of 25-hydroxyvitamin D_3 and 1α,25-dihydroxyvitamin D_3 and their abilities to covalently and specifically modify this pocket. We observed that, although 25-hydroxyvitamin D_3 3β-bromoacetate (25-OH-D_3-3-BE), 1α,25-dihydroxyvitamin D_3 3β-bromoacetate [1α,25$(OH)_2D_3$-3-BE], 1α,25-dihydroxyvitamin D_3 1α-bromoacetate [1α,25$(OH)_2D_3$-1-BE], and 1α,25-dihydroxyvitamin D_3 1α,3β-dibromoacetate [1α,25$(OH)_2D_3$-1,3-di-BE] bound DBP in a specific manner, only [^{3}H]-25-OH-D_3-3-BE and [^{3}H]-1α,-25$(OH)_2D_3$-3-BE affinity labeled the protein. BNPS-skatole cleavages of [^{3}H]-25-OH-D_3-3-BE- and ^{3}H-1α,25$(OH)_2D_3$-3-BE-labeled DBP samples produced the same labeled peptide (N-terminal), demonstrating the specificity of labeling by these analogs. Energy-minimized conformational structures of these bromoacetate derivatives indicated significant changes in the A-ring conformations of these analogs. These structural changes were invoked to explain the inability of [^{3}H]-1α,25$(OH)_2D_3$-1-BE and [^{3}H]-1α,25-$(OH)_2D_3$-1,3-di-BE to affinity label DBP. Overall, these studies suggested that the vitamin D sterol-binding pocket in DBP is sterically quite restrictive. This information could be potentially important in designing future vitamin D-based drugs for several diseases.

Specific binding between a small ligand molecule and its receptor is strongly influenced by the three-dimensional structure of the ligand. In the vitamin D endocrine system, serum vitamin D-binding protein (DBP)[1] binds 25-hydroxyvitamin D_3 (25-OH-D_3) with the highest affinity (K_d = 10^{-11-12} M) (Cooke & Haddad, 1989, 1996; Ray, 1996). This affinity is, however, approximately 1000-fold less for vitamin D_3 and 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], with one less and one more hydroxyl group, respectively, than 25-OH-D_3. On the other hand, the nuclear 1α,25$(OH)_2D_3$ receptor (VDR), present in the 1α,25$(OH)_2D_3$-responsive target cells, has the highest binding affinity for 1α,25$(OH)_2D_3$ (K_d = 10^{-11-12} M). This affinity is much less for 25-OH-D_3 (K_d = 10^{-8-9} M) and virtually nil for vitamin D_3 (Link & DeLuca, 1985). Additionally, binding of 1α,25$(OH)_2D_3$ by VDR is highly stereospecific in nature. For example, 1β,25-dihydroxyvitamin D_3, the synthetic 1-epimer of 1α,-25$(OH)_2$ D_3, displays a very low binding affinity toward VDR. These structural aspects of the molecular recognition/binding by DBP and VDR are of utmost importance, because these binding events are prerequisites for the processing of biological actions, mediated by DBP and VDR.

It is well-recognized that the three-dimensional structure of the ligand-binding pocket of a receptor plays an important role in the ligand-binding process. Thus, a proper understanding of this binding process and its accompanying physiological relevance requires a careful scrutiny of the structural features of the ligand as well as those of the ligand-binding pocket in the corresponding receptor. In the case of DBP, a considerable amount of research has been carried out to probe the vitamin D sterol-binding pocket of this multiligand protein. For example, chemical modification and affinity and photoaffinity labeling studies have been carried out to covalently modify this region (Haddad et al., 1992; Link et al., 1987; Ray et al., 1986, 1991a,b; Swamy et al., 1995a; Swamy & Ray, 1995, 1996). These studies have also demonstrated that vitamin D sterol binding by DBP is restricted to an N-terminal segment of the protein. In the present study, we have investigated the binding and vLBD

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[1] Abbreviations: DBP, vitamin D-binding protein; VDR, vitamin D receptor; 25-OH-D_3, 25-hydroxyvitamin D_3; 1α,25$(OH)_2D_3$, 1α,25-hydroxyvitamin D_3; 25-OH-D_3-3-BE, 25-hydroxyvitamin D_3 3β-bromoacetate; [^{3}H]-25-OH-D_3-3-BE, 25-hydroxy[26(27)-^{3}H]vitamin D_3 3β-bromoacetate; 1α,25$(OH)_2D_3$-3-BE, 1α,25-dihydroxyvitamin D_3 3β-dibromoacetate; [^{3}H]-1α,25$(OH)_2D_3$-3-BE, 1α,25-dihydroxy[1β-^{3}H]-vitamin D_3 3β-dibromoacetate; 1α,25$(OH)_2D_3$-1-BE, 1α,25-dihydroxyvitamin D_3 1α-bromoacetate; [^{3}H]-1α,25$(OH)_2D_3$-1-BE, 1α,25-dihydroxy[1β-^{3}H]vitamin D_3 1α-bromoacetate; 1α,25$(OH)_2D_3$-1,3-di-BE, 1α,25-dihydroxyvitamin D_3 1α,3β-dibromoacetate; [^{3}H]-1α,25$(OH)_2D_3$-1,3-di-BE, 1α,25-dihydroxy[1β-^{3}H]vitamin D_3 1α,3β-dibromoacetate; BNPS-skatole, 3-bromo-3-methyl-2-(2-nitrophenylmercapto)-^{3}*H*-indole. Stereochemistries of the 3- and 1-hydroxyl groups of vitamin D sterols are designated according to cholesterol nomenclature.

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labeling capabilities of several bromoacetate derivatives of 25-OH-D_3 and 1α,25$(OH)_2D_3$ to determine the steric requirements of the vLBD in human serum DBP.

MATERIALS AND METHODS

25-Hydroxy[26(27)-^{3}H]vitamin D_3 ([^{3}H]-25-OH-D_3) (specific activity of 21.6 Ci/mM) was purchased from Amersham Corp. (Arlington Heights, IL). All other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI), except Triton X-100 which was purchased from Sigma Chemical Co. (St. Louis, MO). 25-OH-D_3 and 1α,25-$(OH)_2D_3$ were purchased from Calbiochem (San Diego, CA). DBP was purified from human plasma (American Red Cross, Dedham, MA) by ligand affinity chromatography (Swamy et al., 1995b). UV spectra of various steroid samples were obtained in methanol using a Hitachi 2000 UV/VIS spectrophotometer (San Jose, CA). Energy-minimized three-dimensional conformational structures of various compounds were drawn by MM2 force field calculations with Chem 3 D Plus, Ver 3.1 (1993, Cambridge Scientific Computing, Inc., Cambridge, MA).

25-Hydroxyvitamin D_3 3-bromoacetate (25-OH-D_3-3-BE) and its radiolabeled counterpart, 25-hydroxyvitamin D_3 3β-[26(27)-^{3}H]bromoacetate ([^{3}H]-25-OH-D_3-3-BE) (specific activity of 21.6 Ci/mM), were synthesized according to the published procedure (Swamy & Ray, 1996). 1α,25-Dihydroxyvitamin D_3 3β-bromoacetate [1α,25$(OH)_2D_3$-3-BE], 1α,25-dihydroxyvitamin D_3 1α-bromoacetate [1α,25$(OH)_2D_3$-1-BE], and 1α,25-dihydroxyvitamin D_3 1α,3β-dibromoacetate [1α,25$(OH)_2D_3$-1,3-di-BE] and their radiolabeled counterparts, i.e. 1α,25-dihydroxy[1β-^{3}H]vitamin D_3 3β-bromoacetate [[^{3}H]-1α,25$(OH)_2D_3$-3-BE], 1α,25-dihydroxy[1β-^{3}H]-vitamin D_3 1α-bromoacetate [[^{3}H]-1α,25$(OH)_2D_3$-1-BE], and 1α,25-dihydroxy[1β-^{3}H]vitamin D_3 1α,3β-dibromoacetate [[^{3}H]-1α,25$(OH)_2D_3$-1,3-di-BE] were synthesized according a procedure developed in our laboratory (Ray et al., 1996).

Competitive Radioligand Binding Assays of 25-OH-D_3, 1α,25$(OH)_2D_3$, 25-OH-D_3-3-BE, 1α,25$(OH)_2D_3$-3-BE, 1α,-25$(OH)_2D_3$-1-BE, and 1α,25$(OH)_2D_3$-1,3-di-BE. These assays were carried out according to the published procedure with minor modifications (Swamy & Ray, 1995). In a typical experiment, solutions containing affinity-purified hDBP (200 ng), [^{3}H]-25-OH-D_3 (2000 cpm, 0.1428 pmol), and 25-OH-D_3 (1.16−299.4 pmol) or 25-OH-D_3-3-BE (0.0468−11.99 nmol) or 1α,25$(OH)_2D_3$ (0.1497−19.17 nmol) or 1α,25-$(OH)_2D_3$-3-BE (0.2905−37.2 nmol) or 1α,25$(OH)_2D_3$-1-BE (0.2905−37.2 nmol) or 1α,25$(OH)_2D_3$-1,3-di-BE (1.898−60.742 nmol) in 0.01 mL of ethanol and 0.49 mL of assay buffer (50 mM Tris-HCl, 150 mM sodium chloride, 1.5 mM ethylenediaminetetraacetic acid, and 0.1% Triton X-100 at pH 8.3) were incubated at 4 °C for 20 h followed by treatment with ice-cold Dextran-coated charcoal and centrifugation at 5000*g* at 4 °C. Clear supernatants from the centrifuged samples were mixed with scintillation cocktail and counted for radioactivity. Assays for each sample were carried out in triplicate.

Affinity Labeling Studies of DBP with [^{3}H]-25-OH-D_3-3-BE, [^{3}H]-1α,25$(OH)_2D_3$-3-BE, [^{3}H]-1α,25$(OH)_2D_3$-1-BE, and [^{3}H]-1α,25$(OH)_2D_3$-1,3-di-BE. Samples of DBP (0.02 mg, 0.4 nmol each) in Tris-HCl buffer at pH 8.3 were incubated with either [^{3}H]-25-OH-D_3-3-BE (0.916 pmol), [^{3}H]-1α,25$(OH)_2D_3$-3-BE (1.783 pmol), [^{3}H]-1α,25$(OH)_2D_3$-1-BE (1.689 pmol), or [^{3}H]-1α,25$(OH)_2D_3$-1,3-di-BE (1.56 pmol) in the presence or absence of 25-OH-D_3 (2.5 nmol) (dissolved in 5 μL of ethanol) in a final volume of 0.05 mL. The samples were incubated initially for 12 h at 4 °C to allow the binding of the steroids to the protein followed by 4 h at 25 °C. The samples were heated in a boiling water bath for 5 min with SDS−PAGE sample buffer and electrophoresed on a 10% denaturing polyacrylamide gel. A sample containing prestained standard molecular mass marker proteins was also electrophoresed alongside the experimental samples. After the electrophoresis, the gel was dried and scanned for radioactivity on a Bioscan Imaging Scanner System 2000 (Bioscan Inc., Washington, DC). The positions of standard marker proteins on the gel were located visually.

BNPS-skatole Digestion of DBP Affinity Labeled with [^{3}H]-25-OH-D_3-3-BE and [^{3}H]-1α,25$(OH)_2D_3$-3-BE. BNPS-skatole cleavage of DBP affinity labeled with [^{3}H]-25-OH-D_3-3-BE or [^{3}H]-1α,25$(OH)_2D_3$-3-BE was performed according to the procedure of Fontana (1972). In a typical reaction, 1.0 nmol of DBP was treated with 4.58 pmol of either [^{3}H]-25-OH-D_3-3-BE or [^{3}H]-1α,25$(OH)_2D_3$-3-BE in Tris-HCl buffer at pH 8.3 for 20 h at 4 °C and then for 4 h at 25 °C in a total volume of 0.05 mL. The protein samples were treated with 0.03 mL of water and 10.0 nmol of BNPS-skatole in 0.16 mL of glacial acetic acid for 72 h at 25 °C in the dark. The reaction was terminated by a 3-fold dilution of the reaction mixture with water and extraction with ethyl acetate (3 × 2 mL). The samples were dried in vacuo to remove the acid and were analyzed on SDS−PAGE. A sample containing standard molecular mass marker proteins was also electrophoresed alongside the experimental samples. After the electrophoresis, the gel was dried and scanned for radioactivity.

RESULTS AND DISCUSSION

Competitive radioligand binding assays are traditionally used to determine binding efficiencies of ligands, both natural and synthetic, for their respective receptors. We carried out similar assays of 25-OH-D_3, 1α,25$(OH)_2D_3$, 25-OH-D_3-3-BE, 1α,25$(OH)_2D_3$-3-BE, 1α,25$(OH)_2D_3$-1-BE, and 1α,25-$(OH)_2D_3$-1,3-di-BE (Figure 1A) with human serum DBP. Results of these assays (Figure 1B) demonstrated that 25-OH-D_3 and 1α,25$(OH)_2D_3$, the natural ligands, and their various bromoacetate analogs displaced [^{3}H]-25-OH-D_3, bound to DBP, in a dose-dependent manner. It is noteworthy that modification of the 3β-hydroxyl group of 25-OH-D_3 with a bromoacetate (25-OH-D_3-3-BE) reduced the binding efficiency (of 25-OH-D_3) almost 100-fold. Incorporation of a bromoacetate group at the 3β or 1α position [of 1α,25-$(OH)_2D_3$], however, did not reduce their binding efficiencies quite so drastically as compared to that of 1α,25$(OH)_2D_3$.

Previous studies have shown that [^{3}H]-25-OH-D_3-BE, an affinity labeling analog of 25-OH-D_3, covalently modifies the 25-OH-D_3-binding pocket in DBP (Haddad et al., 1992; Swamy & Ray, 1996). Thus, [^{3}H]-25-OH-D_3-BE provided a basis for comparing labeling capabilities of various bromoacetate derivatives. Incubation of samples of DBP with [^{3}H]-25-OH-D_3-BE, [^{3}H]-1α,25$(OH)_2D_3$-3-BE, [^{3}H]-1α,25$(OH)_2D_3$-1-BE, and [^{3}H]-1α,25$(OH)_2D_3$-1,3-di-BE, either alone or in the presence of an excess of 25-OH-D_3, followed by SDS−PAGE/autoradiographic analysis demon-

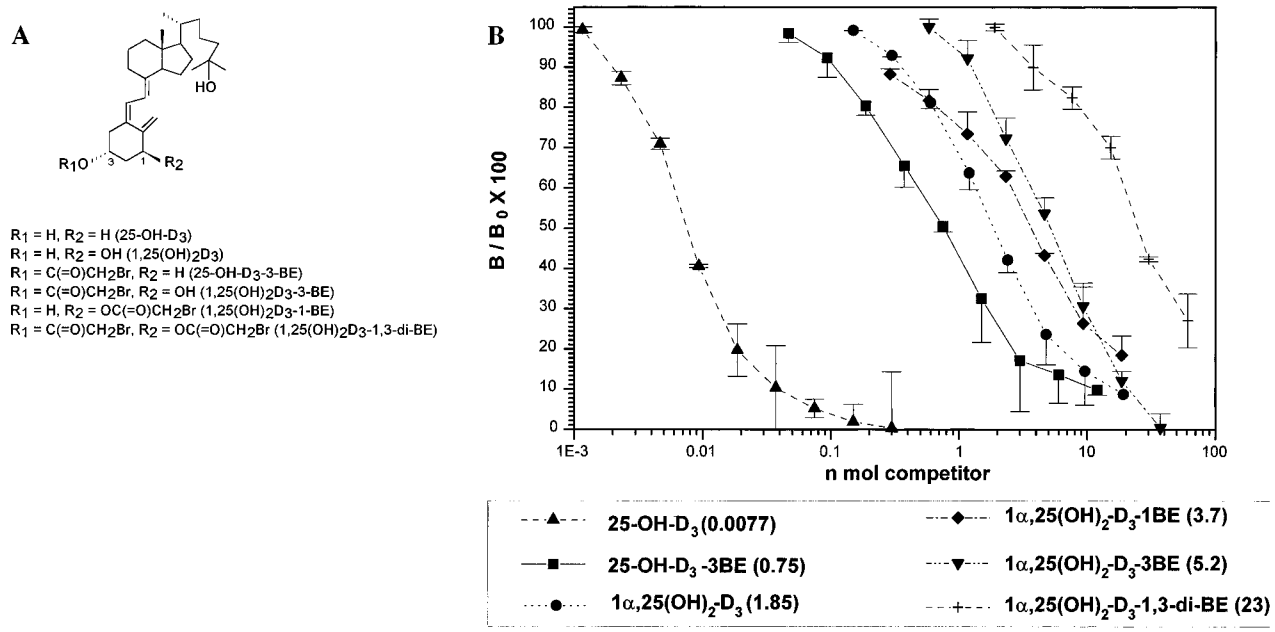

FIGURE 1: (A) Structures of 25-OH-D_3, 1α,25$(OH)_2D_3$, and their bromoacetate derivatives. (B) Competitive radioligand binding assays to determine the binding avidity of 25-OH-D_3-3-BE, 1α,25$(OH)_2D_3$-3-BE, 1α,25$(OH)_2D_3$-1-BE, and 1α,25$(OH)_2D_3$-1,3-di-E to hDBP. A mixture of [^{3}H]-25-OH-D_3 and affinity analogs at different concentrations was incubated with hDBP followed by removal of unbound materials by charcoal stripping and radioactive counting (as described in Materials and Methods). The natural ligands, i.e. 25-OH-D_3 and 1α,25$(OH)_2D_3$, were also used in this comparative study. The values in parentheses represent the concentration of each analog (in nanomolar) needed to achieve 50% inhibition of [^{3}H]-25-OH-D_3 binding by hDBP (the error bars represent standard deviations).

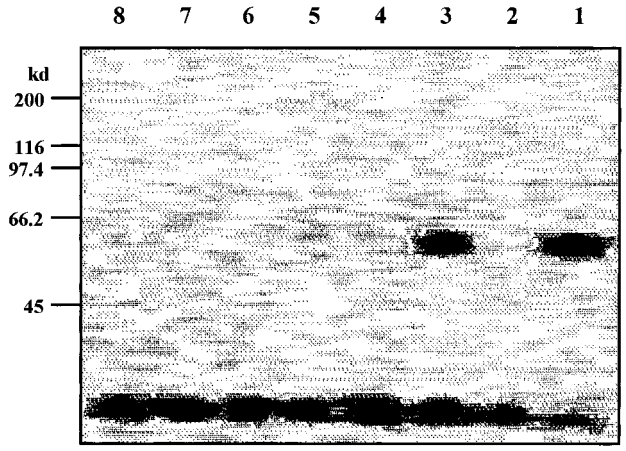

FIGURE 2: Affinity labeling studies of hDBP by 25-OH-D_3-3-BE, 1α,25$(OH)_2D_3$-3-BE, 1α,25$(OH)_2D_3$-1-BE, and 1α,25$(OH)_2D_3$-1,3-di-BE. hDBP (0.4 nmol) was incubated with the affinity analogs as described in the Materials and Methods in the presence or absence of a large molar excess of 25-OH-D_3 (2.5 nmol). The samples were electrophoresed on 10% SDS−PAGE, and radioactivity was analyzed with a Bioscan Imaging Scanner System. Lane 1, DBP + [^{3}H]-25-OH-D_3-3-BE (0.916 pmol). Lane 2, DBP + [^{3}H]-25-OH-D_3-3-BE (0.916 pmol) + 25-OH-D_3 (2.5 nmol). Lane 3, DBP + [^{3}H]-1α,25$(OH)_2D_3$-3-BE (1.783 pmol). Lane 4, DBP + [^{3}H]-1α,25$(OH)_2D_3$-3-BE (1.783 pmol) + 25-OH-D_3 (2.5 nmol). Lane 5, DBP + [^{3}H]-1α,25$(OH)_2D_3$-1-BE (1.689 pmol). Lane 6, DBP + [^{3}H]-1α,25$(OH)_2D_3$-1-BE (1.689 pmol) + 25-OH-D_3 (2.5 nmol). Lane 7, DBP + [^{3}H]-1α,25$(OH)_2D_3$-1,3-di-BE (1.56 pmol). Lane 8, DBP + [^{3}H]-1α,25$(OH)_2D_3$-1,3-di-BE (1.56 pmol) + 25-OH-D_3 (2.5 nmol).

strated that both [^{3}H]-25-OH-D_3-BE and [^{3}H]-1α,25$(OH)_2D_3$-3-BE covalently labeled DBP (Figure 2, lanes 1 and 3, respectively). However, no labeling was observed in the presence of an excess of 25-OH-D_3 in either case (Figure 2, lanes 2 and 4, respectively). Since the protein samples in these experiments consisted of pure DBP (>95% purity as determined by Coomasie blue-staining, results not shown), the radioactive band at the bottom of each lane most probably represented free label. These results strongly indicated that [^{3}H]-25-OH-D_3-BE and [^{3}H]-1α,25$(OH)_2D_3$-3-BE covalently modified the 25-OH-D_3-binding pocket in DBP.

Unambiguous confirmation of the above results was obtained by digesting two DBP samples, labeled with either [^{3}H]-25-OH-D_3-BE or [^{3}H]-1α,25$(OH)_2D_3$-3-BE, with BNPS-skatole and running SDS−PAGE/autoradiographic analysis of the digests. Results of these experiments are shown in Figure 3. BNPS-skatole cleavage of both the samples produced two peptide fragments (approximate molecular masses of 14.5 and 30 kDa) as predicted from the primary structure and positioning of the single Trp (Trp 145) in hDBP (Cooke & David, 1985; Yang et al., 1985; Swamy et al., 1995) [Figure 3A, lanes 2 and 4, respectively, for [^{3}H]-25-OH-D_3-BE and [^{3}H]-1α,25$(OH)_2D_3$-3-BE]. In both the cases, the majority of the radioactivity was associated with the smaller peptide, where M_r = 14.5 kDa [Figure 3B, lanes 2 and 4, respectively, for [^{3}H]-25-OH-D_3-BE and [^{3}H]-1α,-25$(OH)_2D_3$-3-BE]. These results unequivocally proved that both [^{3}H]-25-OH-D_3-BE and [^{3}H]-1α,25$(OH)_2D_3$-3-BE covalently modified the same general area of DBP (N-terminal).

It is generally accepted that vitamin D_3, all its naturally occurring metabolites, and most of their synthetic analogs share a common binding site in DBP. Hence, the above-mentioned results suggested that the gross structure and orientation of both 25-OH-D_3-BE and 1α,25$(OH)_2D_3$-3-BE inside the vitamin D sterol-binding pocket (of DBP) are very similar so that in both cases the carbon atom bearing the electrophile (bromine) is in close proximity with the nucleophilic group(s) of an amino acid residue(s) which is(are) covalently modified by these affinity analogs.

Confirmation of the above possibility was obtained when [^{3}H]-1α,25$(OH)_2D_3$-1-BE failed to label DBP (Figure 2, lanes 5 and 6), although 1α,25$(OH)_2D_3$-1-BE was a stronger DBP binder than 1α,25$(OH)_2D_3$-3-BE (Figure 1). Presumably, placement of the bromoacetate group at the 1α-position [in

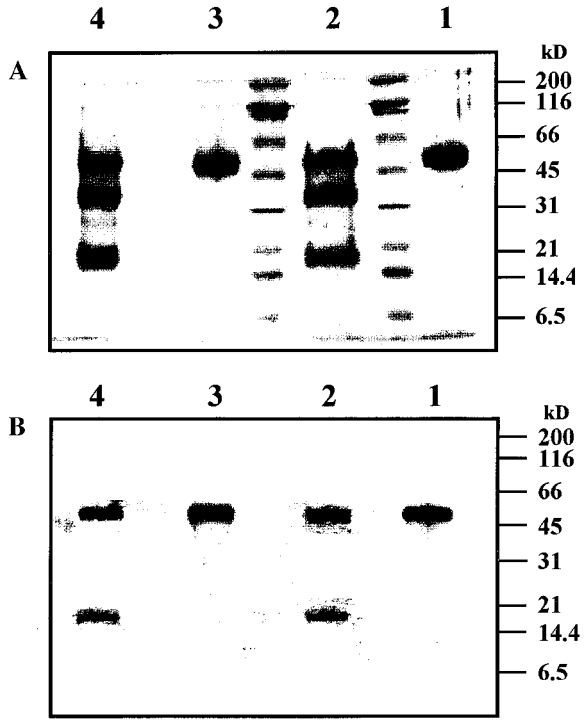

FIGURE 3: BNPS-skatole cleavage of [^{3}H]-25-OH-D_3-3-BE- and [^{3}H]-1α,25$(OH)_2D_3$-3-BE-labeled hDBP. (A) Protein staining. (B) Bioscan imaging for radioactivity. Lane 1, [^{3}H]-25-OH-D_3-3-BE-labeled hDBP. Lane 2, [^{3}H]-25-OH-D_3-3-BE-labeled hDBP + BNPS-skatole. Lane 3, [^{3}H]-1α,25$(OH)_2D_3$-3-BE-labeled hDBP. Lane 4, [^{3}H]-1α,25$(OH)_2D_3$-3-BE-labeled hDBP + BNPS-skatole.

1α,25$(OH)_2D_3$-1-BE] made it inaccessible to the nucleophilic group(s) of the ligand-binding pocket amino acid(s). Hence, no labeling took place.

Our results strongly suggested that "movement" inside the vitamin D sterol-binding pocket in DBP is severely restricted. Otherwise, [^{3}H]-1α,25$(OH)_2D_3$-1-BE could have simply "tumbled" inside the binding pocket and labeled the same amino acid [which was modified by [^{3}H]-1α,25$(OH)_2D_3$-3-BE] or any other amino acid in the vicinity. A striking example of "movement" inside the substrate-binding pocket was observed in the case of estradiol 17β-dehydrogenase (E_2-HSD), where estrone 3-bromoacetate (with an A-ring substituent) and estrone 17-bromoacetate (with a D-ring substituent) affinity labeled the same histidine residue at the active site of the enzyme (Murdock et al., 1988). This observation led to the conclusion that the substrate possibly "rotated" inside the binding pocket, and as a result, bound in two opposing orientations. Comparison of this example with our results clearly demonstrated the sterically restrictive nature of the vitamin D sterol-binding pocket in DBP. These results also indicated that there is no nucleophilic amino acid in close proximity with the 1-bromoacetate group in [^{3}H]-1α,25$(OH)_2D_3$-1-BE.

Further insight into the labeling process was obtained by incubating a sample of DBP with [^{3}H]-1α,25$(OH)_2D_3$-1,3-di-BE. Surprisingly, there was absolutely no labeling in the absence or in the presence of an excess of 25-OH-D_3 (Figure 2, lanes 7 and 8, respectively), despite the fact that this analog carried a bromoacetate group in the 3-position [as in [^{3}H]-25-OH-D_3-BE and [^{3}H]-1α,25$(OH)_2D_3$-3-BE]. Additionally, the DBP binding efficiency of 1α,25$(OH)_2D_3$-1,3-di-BE was only 4.4 times less than that of 1α,25$(OH)_2D_3$-3-BE (Figure 1B). These results suggested that, although [^{3}H]-1α,25-$(OH)_2D_3$-1,3-di-BE could be accommodated inside the vitamin D sterol-binding pocket (in DBP), the carbon atom bearing the leaving atom (bromine) was not in close proximity to a nucleophilic amino acid [possibly the same amino acid which was modified by [^{3}H]-1α,25$(OH)_2D_3$-3-BE].

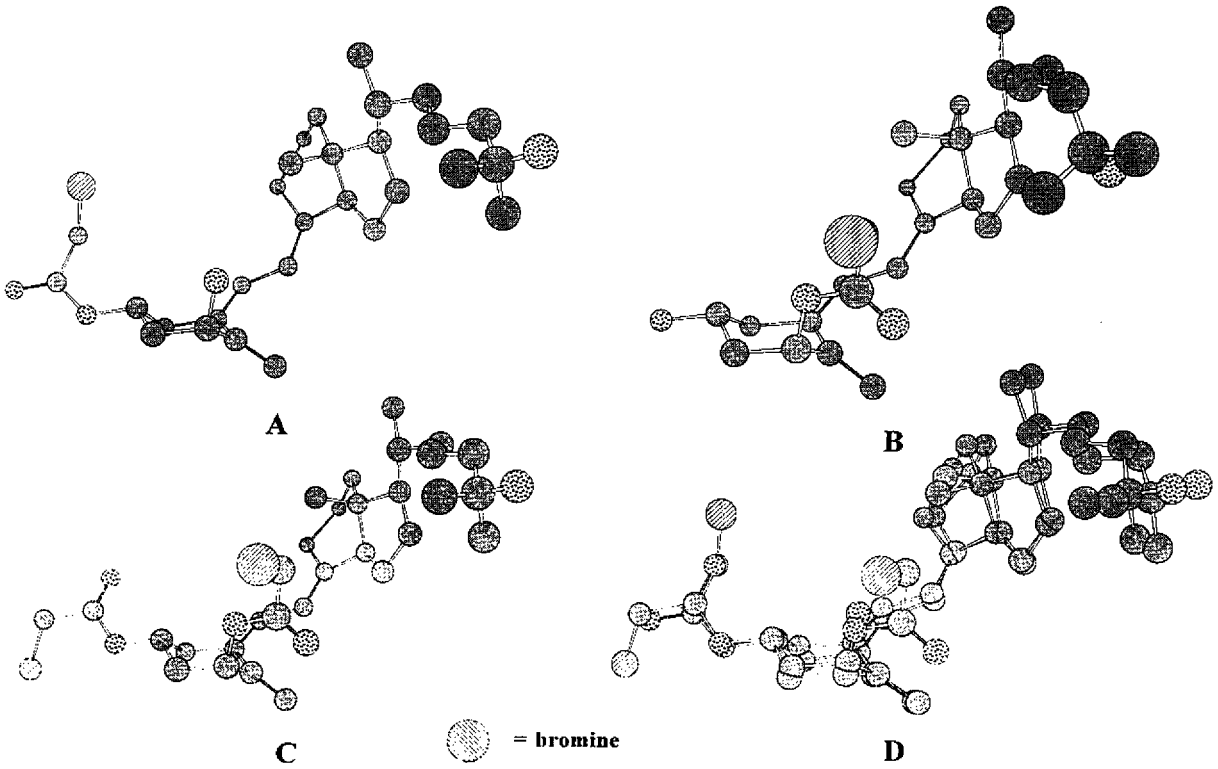

FIGURE 4: Energy-minimized conformational structures of 1α,25$(OH)_2D_3$-3-BE (A), 1α,25$(OH)_2D_3$-1-BE (B), and 1α,25$(OH)_2D_3$-1,3-di-BE (C) and an overlay of panels A and C (D).

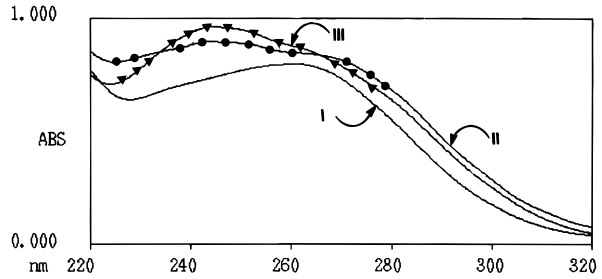

FIGURE 5: UV spectra of 1α,25(OH)$_2$D$_3$-3-BE (I), 1α,25(OH)$_2$D$_3$-1-BE (II), and 1α,25(OH)$_2$D$_3$-1,3-di-BE (III). Overlapping regions of curves II and III are highlighted manually with dots and triangles for clarity.

Energy-minimized three-dimensional structures of the bromoacetates (Figure 4) revealed subtle structural dissimilarities among different analogs, which were also reflected in the UV spectra of these compounds (Figure 5, I−III). Although there is no evidence that DBP binds only the minimum-energy conformers, these "calculated" structures provided simple explanations of certain observations. For example, overlaying of conformational structures of 1α,25(OH)$_2$D$_3$-3-BE and 1α,25(OH)$_2$D$_3$-1,3-di-BE projected the bromine atoms in opposite directions (Figure 4D). This could possibly explain why [^{3}H]-1α,25(OH)$_2$D$_3$-3-BE, with the bromine atom in close proximity to a nucleophilic amino acid, covalently labeled DBP, but [^{3}H]-1α,25(OH)$_2$D$_3$-1,3-di-BE did not.

DBP in the serum is responsible for scavenging vitamin D sterols, including 1α,25(OH)$_2$D$_3$ and its synthetic analogs, and possibly translocating them to target tissues. Hence, close scrutiny of the interaction between DBP and these compounds is warranted. This is particularly important in light of the discovery that 1α,25(OH)$_2$D$_3$ and several of its synthetic analogs possess antiproliferative/anticancer properties (Bouillon et al., 1995). Hence, information about the structures of the ligand and the ligand-binding pocket will be important in developing the next generation of therapeutic agents based on 1α,25(OH)$_2$D$_3$. It is noteworthy in this context that affinity labeling agents were originally developed as active site-directed irreversible inhibitors of enzymes as new tools to combat cancer (Sweet & Murdock, 1987). Finally, since DBP binds most of the metabolites of vitamin D$_3$ and their synthetic analogs, it may be possible in future to extrapolate the results obtained in the present studies to other vitamin D sterol-binding proteins and enzymes.

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REFERENCES

Bouillon, R., Okamura, W. H., & Norman, A. W. (1995) *Endocr. Rev. 16*, 200−257.
Cooke, N. E., & David, E. V. (1985) *J. Clin. Invest. 76*, 2420−2424.
Cooke, N. E., & Haddad, J. G. (1989) *Endocr. Rev. 10*, 294−307.
Cooke, N. E., & Haddad, J. G. (1996) *Endocr. Rev. 4*, 125−128.
Fontana, A. (1972) *Methods Enzymol. 25*, 419−423.
Haddad, J. G., Hu, Y. Z., Kowalski, M. A., Laramore, C., Ray, K., Robzyk, P., & Cooke, N. E. (1992) *Biochemistry 31*, 7174−7181.
Link, R., & DeLuca, H. F. (1985) in *The Receptors* (Conn, P. M., Ed.) Vol. II, pp 1−35, Academic Press, Orlando, FL.
Link, R., Kutner, A., Schnoes, H. K., & DeLuca, H. F. (1987) *Biochemistry 26*, 3957−3964.
Murdock, G. L., Warren, J. C., & Sweet, F. (1988) *Biochemistry 27*, 4452−4458.
Ray, R. (1996) *Proc. Soc. Exp. Biol. Med. 212*, 305−312.
Ray, R., Holick, S. A., Hanafin, N., & Holick, M. F. (1986) *Biochemistry 25*, 4729−4733.
Ray, R., Bouillon, R., Van Baelen, H. G., & Holick, M. F. (1991a) *Biochemistry 30*, 4809−4813.
Ray, R., Bouillon, R., Van Baelen, H. G., & Holick, MF (1991b) Biochemistry 30: 7638−7642.
Swamy, N., & Ray, R. (1995) *Arch. Biochem. Biophys. 319*, 504−507.
Swamy, N., & Ray, R. (1996) *Arch. Biochem. Biophys. 333*, 139−144.
Swamy, N., Brisson, M., & Ray, R. (1995a) *J. Biol. Chem. 270*, 2636−2639.
Swamy, N., Roy, A., Chang, R., Brisson, M., & Ray, R. (1995b) *Protein Expressionn Purif. 6*, 185−188.
Sweet, F., & Murdock, G. L. (1987) *Endocr. Rev. 8*, 154−184.
Yang, F., Brune, J. I., Naylor, S. L., Cupples, R. L., Naberhaus, K. H., & Bowman, B. H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7994−7998.

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