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Photoaffinity Labeling of Serum Vitamin D Binding Protein by 3-Deoxy-3-azido-25-hydroxyvitamin D_3†

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ABSTRACT: 3-Deoxy-3-azido-25-hydroxyvitamin D_3 was covalently incorporated in the 25-hydroxyvitamin D_3 binding site of purified human plasma vitamin D binding protein. Competition experiments showed that 3-deoxy-3-azido-25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_3 bind at the same site on the protein. Tritiated 3-deoxy-3-azido-25-hydroxyvitamin D_3 was synthesized from tritiated 25-hydroxyvitamin D_3, retaining the high specific activity of the parent compound. The tritiated azido label bound reversibly to human vitamin D binding protein in the dark and covalently to human vitamin D binding protein after exposure to ultraviolet light. Reversible binding of tritiated 3-deoxy-3-azido-25-hydroxyvitamin D_3 was compared to tritiated 25-hydroxyvitamin D_3 binding to human vitamin D binding protein. Scatchard analysis of the data indicated equivalent maximum density binding sites with a $K_{D,app}$ of 0.21 nM for 25-hydroxyvitamin D_3 and a $K_{D,app}$ of 1.3 nM for the azido derivative. Covalent binding was observed only after exposure to ultraviolet irradiation, with an average of 3% of the reversibly bound label becoming covalently bound to vitamin D binding protein. The covalent binding was reduced 70–80% when 25-hydroxyvitamin D_3 was present, indicating strong covalent binding at the vitamin D binding site of the protein. When tritiated 3-deoxy-3-azido-25-hydroxyvitamin D_3 was incubated with human plasma in the absence and presence of 25-hydroxyvitamin D_3, 12% of the azido derivative was reversibly bound to vitamin D binding protein. After ultraviolet irradiation, four plasma proteins covalently bound the azido label, but vitamin D binding protein was the only protein of the four that was unlabeled in the presence of 25-hydroxyvitamin D_3.

Vitamin D and its hydroxylated metabolites are transported in serum on a specific vitamin D binding protein (DBP).[1] DBP, which is identical with group-specific component (Bouillon et al., 1976; Daiger et al., 1975; Haddad & Walgate, 1976), is an abundant and multifunctional glycoprotein (Haddad, 1984). In addition to binding vitamin D, it can also bind monomers of actin (Cooke et al., 1979; Van Baelen et al., 1980) and associate with membranes of a variety of cell types, including B-lymphocytes (Petrini et al., 1983a,b) and

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[1] Abbreviations: DBP, vitamin D binding protein; hDBP, human vitamin D binding protein; 25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2$-D_3, 1,25-dihydroxyvitamin D_3; Az-25-OH-D_3, 3-deoxy-3-azido-25-hydroxyvitamin D_3; BSA, bovine serum albumin; HDL, high-density lipoproteins; EDTA, ethylenediaminetetraacetic acid; HA, hydroxylapatite; Me_2SO, dimethyl sulfoxide; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

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T-lymphocytes (Petrini et al., 1985). DBP has been isolated and characterized from several species (Bouillon et al., 1976, 1978, 1980; Haddad & Walgate, 1976; Imawari et al., 1976). The complete amino acid sequence of human DBP has been determined, providing evidence for strong homology with albumin and α-fetoprotein, including 3-fold internal homology (Cooke & David, 1985; Schoentgen et al., 1986; Yang et al., 1985).

The association of vitamin D metabolites with DBP is specific and of high affinity, with one sterol binding site per molecule (Haddad & Walgate, 1976). The affinities for DBP vary among the metabolites, with 25-hydroxyvitamin D_3 (25-OH-D_3), the major circulatory form of the vitamin, having one of the highest affinities for the protein (Haddad, 1984).

The use of photoaffinity labels can be a powerful technique fory studying the properties and functions of proteins. The first photoactivatable analogues of 25-OH-D_3 and 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2$-D_3] have been prepared by Kutner et al. (1986) and Ray et al. (1985a,b, 1986). The series of photoactivatable analogues of 25-OH-D_3 that were prepared in this laboratory were assayed for their activity in binding to DBP (Kutner et al., 1986). The C-3 hydroxyl was chosen as the place for introduction of the photoactivatable group, since removal or derivatization of this hydroxyl results in only a slight reduction of binding affinity (Belsey et al., 1974). The most effective vitamin D label having the highest binding affinity for DBP was 3-deoxy-3-azido-25-hydroxyvitamin D_3 (Az-25-OH-D_3). Az-25-OH-D_3, which showed only a 20-fold reduction in binding affinity compared to the parent compound, has the photoactivatable azido substituent attached directly to the C-3 position.

In this paper we present the synthesis of the first tritiated photoactivatable vitamin D analogue, Az-25-OH-[26,27-^{3}H]D_3, and demonstrate the covalent incorporation of the label at the 25-OH-D_3 binding site of purified human DBP. This work was presented at the ASBC meeting (Link et al., 1986a), and a preliminary report of similar work appeared while this paper was in preparation (Ray et al., 1986).

Materials and Methods

Materials. Vitamin D was purchased from Aldrich (Milwaukee, WI), and 25-OH-D_3 was a gift from the Organon Co. (West Orange, NJ). 25-OH-[26,27-^{3}H]D_3 (160 Ci/mmol) was prepared by a previously published procedure (Napoli et al., 1980) and supplied by Du Pont/New England Nuclear (Boston, MA). All other chemicals were of the best quality available from commercial sources. Human DBP was purified from human plasma obtained from the American Red Cross (Madison, WI), by a previously published procedure (Link et al., 1986b).

Synthesis of Az-25-OH-[26,27-^{3}H]D_3. All manipulations involving the use of Az-25-OH-[26,27-^{3}H]D_3 were carried out under low-intensity yellow light unless otherwise stated.

A solution of 25-OH-[26,27-^{3}H]D_3 (320 μCi, specific activity 160 Ci/mmol) in toluene–ethanol was dried down under reduced pressure, and traces of ethanol were removed by the addition and evaporation of an addition 20 mL of dry toluene. To the residue, redissolved in 1 mL of dry pyridine (1 mL), vitamin D_3 (10 mg) was added, followed by 15 mg of recrystallized *p*-toluenesulfonyl chloride. The mixture was stirred under nitrogen at ambient temperature for 42 h, and then an additional 2 mg of *p*-toluenesulfonyl chloride was added. After an additional 17 h of stirring at room temperature, the reaction was shown to be complete by TLC (system A). The mixture was quenched with ice-cold saturated $NaHCO_3$. Stirring was continued for 30 min, and the mixture was extracted with ethyl ether. The organic phase was washed with 5% $NaHCO_3$, 5% HCl, water, saturated $CuSO_4$, and saturated NaCl and dried over $MgSO_4$. Magnesium sulfate was filtered off, and the solvent was evaporated in a stream of N_2. Crude tosylate thus obtained was used for the next step without further purification. The oily residue was dissolved in 100 μL of dry Me_2SO, and the solution of sodium azide (3.2 mg) in 100 μL of Me_2SO was added. The mixture was stirred at 60 °C under nitrogen in the dark until a homogeneous solution was obtained. Stirring was continued for an additional 17 h at 45 °C until no starting tosylate was detected by TLC using the solvent system B for detection of vitamin D_3 carrier and system A for radioactive products. The mixture was diluted with 500 μL of water and 300 μL of ethyl ether and stirred for 15 min. The water phase was extracted with ethyl ether, and combined organic layers were washed with water and saturated NaCl and dried over $MgSO_4$. Ethyl ether was replaced with 400 μL of 7% ethyl acetate in hexane, and the solution was filtered through a silica gel Sep-Pak cartridge (Waters Associates, Milford, MA) followed by 20 mL of 3% propanol-2 in hexane. The filtrate was evaporated and the residue redissolved in 200 μL of 7% ethyl acetate in hexane. The solution was injected in one portion into a Zorbax-Sil (Du Pont/NEN) HPLC column (4.6 × 250 mm) eluted with 7% ethyl acetate in hexane at a flow rate of 2 mL/min. Fraction numbers 28 and 29 (1 mL each) containing the product were pooled, evaporated, and dissolved in 1 mL of toluene–ethanol for storage. The partially purified material contained a single radioactive product and multiple nonradioactive contaminants. After 1 year of storage at −70 °C, the radioactive product was 85% pure.

Highly purified product, in which the nonradioactive contaminants were removed, was achieved by two additional runs on a Zorbax-Sil column. A portion of the semipure material was first run in 0.4% propanol-2 in hexane, followed by a run in 3.5% ethyl acetate in hexane. The product stored in the final column solvent was stable for at least 1 month.

TLC was performed on precoated aluminum silica gel sheets with UV indicator (60 F254) in solvent systems A, hexane–ethyl acetate, 1:1 (v/v), and B, hexane–ethyl acetate, 95:5. Radioactive compounds were scanned directly from the plates by using a Bioscan System 200 imaging scanner (Bioscan, Inc., Washington, D.C.).

HPLC was carried out on a Waters Associates liquid chromatograph equipped with a Model 6000A solvent-delivery system, a Model 6UK universal injector, and a Model 450 variable-wavelength detector.

Photolysis. The samples to be photoactivated were exposed to shortwave (254-nm) UV light from a Minerallight lamp, Model R-52G. The lamp was placed at a distance of 5 cm above the samples (200–250 μL), which were mixing in vials (1.3 × 5 cm) in an ice bath.

Binding Studies. All experiments were performed in the dark or under yellow lights. For the competition and direct binding studies, the hDBP was diluted in PBS and 0.25% BSA, and 250 μL of the protein solution was added to 20 μL of ethanol containing the tritiated sterol in the absence or presence of nonradioactive 25-OH-D_3 or Az-25-OH-D_3. These samples were incubated overnight on ice prior to assaying for reversible binding.

For the photoactivation experiments with purified hDBP, the hDBP was diluted to 200 nM in PBS and added to an ethanol solution (6–8%) containing tritiated sterol (0.47–2.0 nM) in the absence or presence of nonradioactive 25-OH-D_3 (5 μM). For the experiments with human plasma, the plasma was diluted 30-fold in PBS, for a final DBP concentration of

approximately 150 nM, and added to an ethanol solution (6–8%) containing tritiated sterol (1.4–2.0 nM) in the absence or presence of nonradioactive 25-OH-D_3 (5 μM. These samples were incubated overnight on ice prior to photoactivation. Photolysis was carried out as described above, and samples were assayed for reversible and covalent binding. Samples were also prepared for gel electrophoresis.

Specific binding is considered to be the difference between total binding in the absence of nonradioactive 25-OH-D_3 and nonspecific binding in the presence of nonradioactive 25-OH-D_3.

Reversible Binding Assay/HA Assay. Reversibly bound tritiated sterol was determined by hydroxylapatite assay (HA) assay described by Williams and Gorski (1974) and modified by Wecksler and Norman (1979). An equal volume or 0.1 mL of HA [a 50% suspension in TE buffer (50 mM Tris-HCl and 1.5 mM EDTA, pH 7.4)] was added to the samples and incubated on ice for 20 min. The HA was pelleted by centrifugation, and the pellets were washed with 2 × 1 mL of TE and 0.5% Triton X-100. The pellets were transferred to mini scintillation vials with 2 × 0.25 mL of 95% ethanol, and 3 mL of Scint A (Packard Instrument Co., Downers Grove, IL) was added to each vial. The radioactivity was determined by liquid scintillation counting in a Prias PLD Tri-Carb minivial counter (Packard, Downers Grove, IL).

Covalent Binding Assay/Filter Assay. The amount of covalently bound tritiated sterol was determined by a filter assay based on an assay by Katzenellenbogen et al. (1975), modified for use with DBP and vitamin D compounds. To assay for covalently bound tritiated sterol, 25-μL samples were mixed with 25 μL of filter buffer (PBS, 2.3% SDS, and 5% 2-mercaptoethanol) and incubated at 37 °C for 1 h. As a carrier protein, 5 μL of β-lactoglobulin (5 mg/mL) was added to the samples, and a 40-μL aliquot was spotted on a Whatman filter disk (3 mm, 2.3 cm) and allowed 15–30 min to dry. The filters were washed in 95% ethanol, chloroform–methanol (1:1), and ethyl acetate, two washes each at 10 mL/disk. The disks were air-dried and placed in a mini scintillation vial, mixed with 3 mL of Scint A, and counted.

Gel Electrophoresis. One-dimensional (SDS–polyacrylamide) gel electrophoresis was carried out by the method of Laemmli (1970), with a 0.75-mm discontinuous slab gel containing a 10% acrylamide separating gel and a 4.75% acrylamide stacking gel. Two-dimensional gels were performed according to the method of O'Farrell (1975) by Kendrick Laboratory (Madison, WI). The isoelectric focusing pH gradient extended from pH 4.1 to pH 8.3, and the second dimension was a 10% acrylamide slab gel. The gel samples were prepared by incubating 125–150 μL of the labeled protein with 5% 2-mercaptoethanol for 1 h at 25 °C, then adding 50 μg of β-lactoglobulin, and precipitating the proteins with 2 volumes of cold ethanol. The SDS–polyacrylamide gel electrophoresis, the precipitate was resuspended in SDS sample buffer and boiled for 5 min. The above samples were diluted with an equal volume of lysis buffer for the two-dimensional electrophoresis gels.

For fluorography the gels were treated with ENHANCE (Du Pont/NEN) according to the manufacturer's instructions. The dried gel was exposed to preflashed X-Omat AR-5 X-ray films (Kodak) at −80 °C. Films were developed in GBX developer and fixed in GBX fixer (Kodak).

RESULTS

Synthesis of Az-25-OH-[26,27-^{3}H]D_3. Az-25-OH-[26,27-^{3}H]D_3 used as a photoaffinity radiolabel was synthesized from 25-OH-[26,27-^{3}H]D_3 by the method previously developed in this laboratory (Kutner et al., 1986). Preparation of the radiolabel was carried out with the use of vitamin D_3 added to the radioactive substrate as a carrier substance with facilitate the monitoring of the preparative procedure not only by scanning the radioactive compounds but also by simple running of standard TLC plates. The use of the carrier was found to be necessary, as the time of the reactions run in a microscale was as expected much longer than that of the previously reported multimilligram scale. The carrier was removed at the final purification step of the radioactive product. This way the radioactive product retained the high specific activity of the substrate.

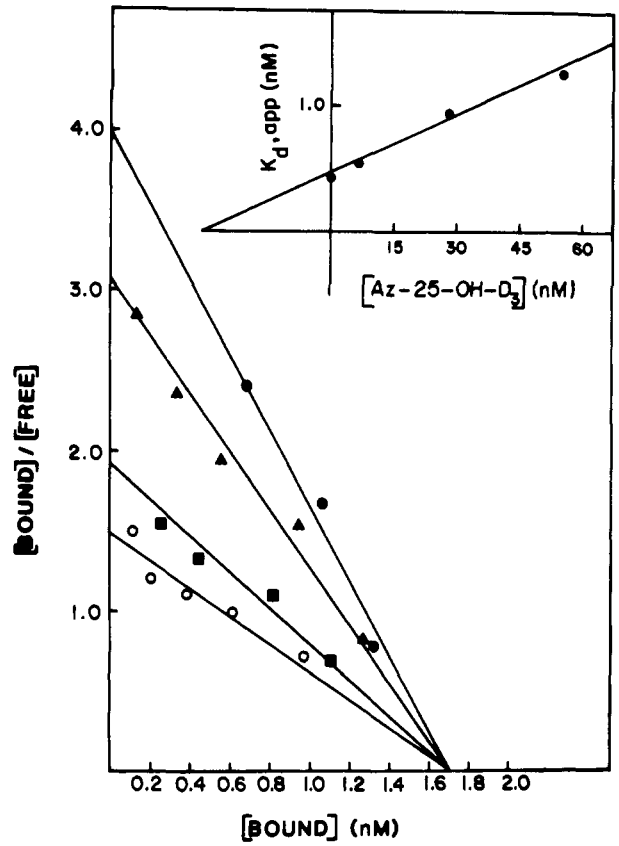

FIGURE 1: Inhibition of 25-OH-[26,27-^{3}H]D_3 binding to hDBP by Az-25-OH-D_3 (in the dark). hDBP (72 ng/mL) in TES (50 mM Tris, 1.5 mM EDTA, 150 mM NaCl, pH 7.4) containing 0.25% BSA was mixed with 25-OH-[26,27-^{3}H]D_3 (0.2–2 nM) in ethanol in the absence of (●) or the presence of (Az-25-OH-D_3) at 7 nM (△), 28 nM (■), or 55 nM (○). The mixture was incubated for 18 h at 0 °C in the dark. Bound 25-OH-[26,27-^{3}H]D_3 was determined by HA assay. Nonspecific binding was determined in the presence of 750 nM 25-OH-D_3. Points represent the mean of duplicate determinations. (Inset) Plot of $K_{D,app}$ vs. Az-25-OH-D_3 provided a K_I of 30 nM.

Az-25-OH-D_3 Binding to hDBP in the Dark. The ability of Az-25-OH-D_3 to inhibit 25-OH-[26,27-^{3}H]D_3 binding to hDBP in the dark was studied. 25-OH-[26,27-^{3}H]D_3 binding to hDBP was monitored in the absence and presence of constant concentrations of Az-25-OH-D_3. The nonspecific binding of 25-OH-[26,27-^{3}H]D_3 to hDBP, determined in the presence of excess nonradioactive 25-OH-D_3, was subtracted from the data. Scatchard analysis of the data (Figure 1) showed a competitive inhibition pattern in which Az-25-OH-D_3 decreased 25-OH-[26,27-^{3}H]D_3 binding to hDBP without affecting the maximum number of binding sites. Under the conditions of the experiment, 24% of the added AZ-25-OH-D_3 was soluble, determined from the recovery of tritium in parallel samples of Az-25-OH-[26,27-^{3}H]D_3-labeled stock Az-25-OH-D_3. The concentration of Az-25-OH-D_3 in Figure 1 represents the amount of soluble azido label. a plot of the

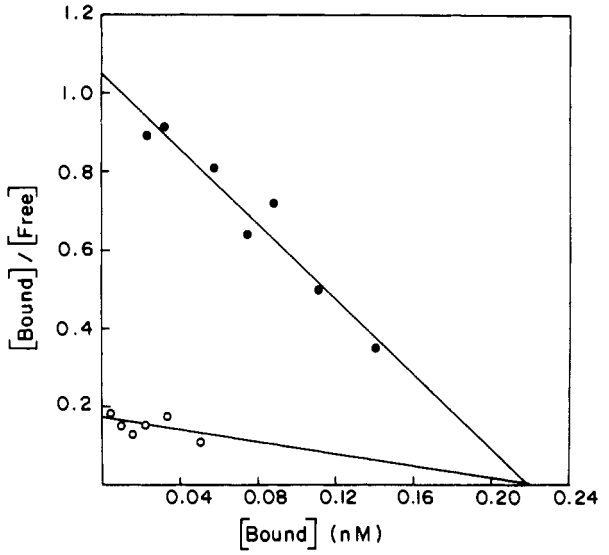

FIGURE 2: Scatchard plot of the binding data of Az-25-OH-[26,27-^{3}H]D_3 and 25-OH-[26,27-^{3}H]D_3 to hDBP. hDBP (12 ng/mL) in PBS and 0.25% BSA was incubated with Az-25-OH-[26,27-^{3}H]D_3 (0.02–0.5 nM) (○) or 25-OH-[26,27-^{3}H]D_3 (0.02–0.5 nM) (●) for 18 h at 0 °C in the dark. Tritiated sterol bound to hDBP was determined by HA assay. The data provided a $K_{D,app}$ of 1.3 nM for Az-25-OH-[26,27-^{3}H]D_3 and a $K_{D,app}$ of 0.21 nM for 25-OH-[26,27-^{3}H]D_3 with a B_{max} of 0.22 nM.

$K_{D,app}$ vs. the concentration of soluble Az-25-OH-D_3 (Figure 1, inset) provided a K_I of 30 nM.

The reversible binding of Az-25-OH-[26,27-^{3}H]D_3 to hDBP in the dark was compared to 25-OH-[26,27-^{3}H]D_3 binding to hDBP. Unfortunately, the conditions that provide good data for 25-OH-D_3 binding to hDBP did not work well for the azido compound. Because of the low aqueous solubility of vitamin D compounds, a detergent or a protein is required to maintain the vitamin in solution. In the presence of Triton X-100, there is no detectable binding of the Az-25-OH-[26,27-^{3}H]D_3. In the presence of BSA as a carrier protein, a high level of nonlinear binding was observed in the presence of excess 25-OH-D_3, indicating binding of the azido compound to albumin. Furthermore, these conditions, which are the same as those employed for the inhibition experiment, showed only 24% of the added Az-25-OH-[26,27-^{3}H]D_3 to be soluble.

An estimation of direct binding of the photolabel to hDBP was achieved by monitoring the total binding at low concentrations of Az-25-OH-[26,27-^{3}H]D_3, where the nonspecific binding is low. The soluble concentrations of the tritiated vitamin and azido compounds were used for the calculations in Figure 2. A Scatchard plot of these data and data from a parallel experiment with 25-OH-[26,27-^{3}H]D_3 (Figure 2) showed an equivalent number of maximum binding sites and a 6-fold higher apparent K_D. The $K_{D,app}$ for 25-OH-D_3 was 0.21 nM, and the $K_{D,app}$ for Az-25-OH-D_3 was 1.3 nM.

Stability of hDBP to UV Irradiation. Activation of Az-25-OH-D_3 requires medium-intensity, short-wavelength (254-nm) irradiation. The stability of hDBP to these UV-irradiation conditions was monitored in regard to 25-OH-[26,27-^{3}H]D_3 binding. A time course of UV irradiation of free hDBP and sterol–protein complex is seen in Figure 3. The free hDBP, which was assayed for 25-OH-[26,27-^{3}H]D_3 binding activity after irradiation, showed retention of approximately 70% of the sterol binding activity after 3–5 min of irradiation. Irradiation of the 25-OH-[26,27-^{3}H]D_3–hDBP complex showed that approximately 40% of the sterol–protein complexes were retained after 3–5 min of irradiation. These results indicate that both the protein and the vitamin D

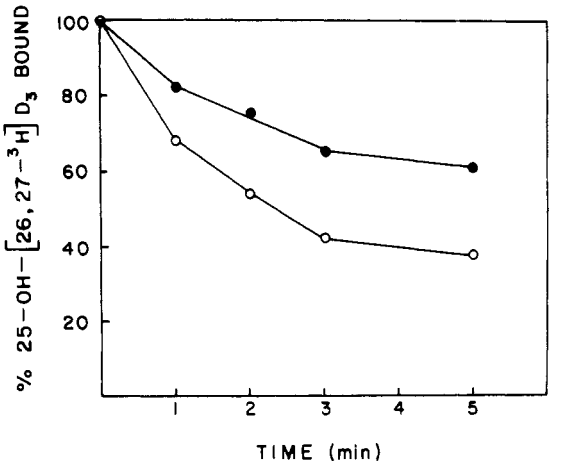

FIGURE 3: Time course of the stability of 25-OH-D_3 binding to hDBP under the UV-irradiation conditions required for photoactivation of Az-25-OH-D_3. hDBP (7 μg/mL) in 50 mM Tris, 1.5 EDTA, and 150 mM NaCl (TES) was exposed to shortwave (254-nM) UV light in the absence of ligand (●) or in the presence of 2.8 nM 25-OH-[26,27-^{3}H]D_3 (○) (complex formed by incubation at 0 °C for 18 h). The unliganded hDBP was assayed after UV irradiation by incubating a $^1/_{100}$ dilution in TES and 0.25% BSA of each sample with 1 nM 25-OH-[26,27-^{3}H]D_3 for 18 h at 0 °C and by using the HA assay to determine bound 25-OH-[26,27-^{3}H]D_3. The retention of ligand by the 25-OH-[26,27-^{3}H]D_3–hDBP complex was determined by HA assay. The amount of bound ligand in samples not exposed to UV light was taken as 100%.

structures were altered by the UV irradiation. Therefore, irradiation times of 5 min or less were used in subsequent experiments with the azido label to minimize the nonspecific covalent binding.

Covalent Binding of Azido Label to hDBP. In order to incorporate covalently Az-25-[26,27-^{3}H]D_3 into the 25-OH-D_3 binding site of hDBP, the photolabel would have to be positioned in the sterol binding site prior to irradiation, retain this position, and become covalently attached upon UV activation. Maximum reversible binding of the label was achieved by having the protein in large excess in the incubation mixture. Generally a 100:1 molar ratio of hDBP to photolabel was used. This level of hDBP also provided a protein concentration sufficient to solubilize the vitamin D compounds. Therefore, the reactions did not require the use of detergents or carrier protein to maintain the label in solution. A control for nonspecific binding was included in which excess 25-OH-D_3 was present. In this case hDBP was at 200 nM, Az-25-OH-[26,27-^{3}H]D_3 was at 2 nM, and 25-OH-D_3 was at 5 μM.

A time course of short-wavelength UV irradiation was performed on the Az-25-[26,27-^{3}H]D_3–hDBP complex. Reversible binding and covalent binding of photolabel were monitored in the absence and presence of excess 25-OH-D_3 (Figure 4). The initial reversible binding assays showed 21% of the label was bound to hDBP after the overnight incubation in the dark. The level of reversibly bound ligand decreased with increasing times of UV irradiation. The pattern was similar to that seen for retention of the sterol–protein complex seen in Figure 3. There appeared to be slightly greater retention of the azido–protein complex after UV irradiation, as would be expected if covalent binding occurred. In the presence of excess 25-OH-D_3, 94% of the Az-25-OH-[26,27-^{3}H]D_3 was displaced from the protein and a slight increase was observed in the nonspecific binding with exposure to UV light.

One method used to monitor covalent binding of the photolabel to hDBP was a filter assay. In this assay the labeled protein is reduced and denatured in a buffer containing SDS

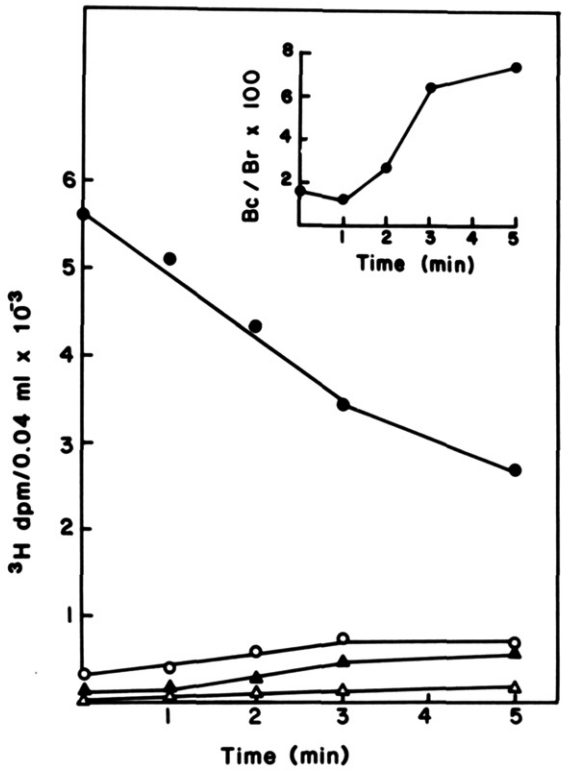

FIGURE 4: Time course of reversible binding and covalent binding of Az-25-[26,27-^{3}H]D_3 to hDBP with exposure to shortwave (254-nm) UV irradiation. hDBP (10 μg/mL) in PBS, pH 7.4, was incubated with 2 nM Az-25-[26,27-^{3}H]D_3 for 18 h at 0 °C, in the absence or presence of excess 25-OH-D_3 in the dark. Samples (250 μL) were irradiated for the indicated period of time and assayed for retention of reversibly and covalently bound Az-25-OH-[26,27-^{3}H]D_3. Reversibly bound label was determined by HA assay of hDBP in the absence (●) or presence (○) of 25-OH-D_3. Covalently bound label was determined by filter assay of hDBP in the absence (▲) or presence (△) of 25-OH-D_3. (Inset) The percent of covalently bound Az-25-OH-[26,27-^{3}H]D_3 (Bc) of the reversibly bound label prior to irradiation (Br) with length of time of UV irradiation.

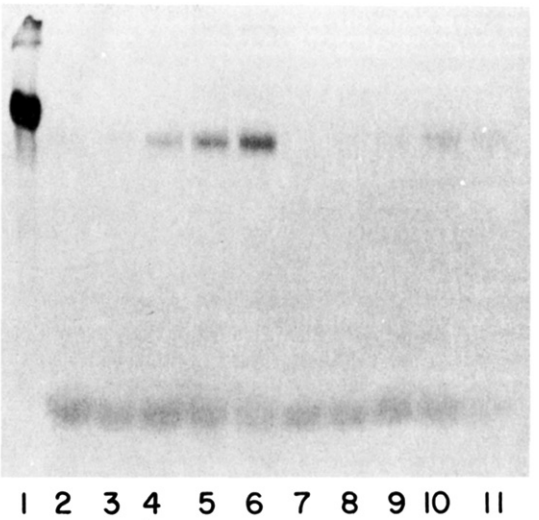

FIGURE 5: Fluorogram of the time course of UV irradiation of Az-25-OH-[26,27-^{3}H]D_3 binding to hDBP. The fluorogram represents an 8-week exposure to X-ray film of a 10% SDS–polyacrylamide gel. Lane 1 contains [^{14}C]BSA as a molecular weight marker. Samples of Az-25-OH-[26,27-^{3}H]D_3 bound to hDBP in the absence of 25-OH-D_3 are in lanes 2–6 and in the presence of 25-OH-D_3 are in lanes 7–11. The time of UV irradiation was 0 min for lanes 2 and 7, 1 min for lanes 3 and 8, 2 min for lanes 4 and 9, 3 min for lanes 5 and 10, and5 min for lanes 5 and 11.

and 2-mercaptoethanol before spotting onto the filters. The filters are washed with a series of organic solvents to remove the noncovalently attached sterol. The amount of covalently bound tritiated label for the time course is also seen in Figure 4. After 2 min of UV irradiation there was a detectable amount of covalently bound label. This amount increased at 3 min and began to taper off at 5 min. The samples that contained excess 25-OH-D_3 showed a very slight increase in bound label with UV irradiation. The difference between the sample in the absence or presence of 25-OH-D_3 was considered specific covalently bound label (Bc). The inset in Figure 4 shows the percent of reversibly bound label prior to UV irradiation (Br) that becomes specific covalent bound label (Bc/Br) with time of irradiation. In the absence of light or after 1 min of UV light, 1–1.5% of the reversibly bound label appears to be covalently bound. The maximum amount of covalent incorporation is achieved after 3–5 min of UV light, with 6–7% of the reversibly bound label becoming covalently bound.

A second method used to monitor covalent incorporation of the azido label into hDBP was to prepare a fluorogram of an SDS gel of the UV-irradiated samples. Figure 5 represents a fluorogram of the samples from the time course presented in Figure 4. A long exposure of the film to the gel represented here shows low nonspecific binding of label to hDBP and the very evident increase in covalent binding of label with increasing time of irradiation. The tritium incorporation into hDBP was evident after a 1-week exposure of film to gel for the 2-, 3-, and 5-min time points with no apparent nonspecific binding. The labeling of a low molecular weight protein seen only with long exposure represents azido binding to a carrier protein added after the UV irradiation. This secondary labeling was eliminated by adding 2-mercaptoethanol to the samples prior to the addition of carrier protein.

As a control, a parallel experiment was conducted of a time course of UV irradiation on the 25-OH-[26,27-^{3}H]D_3–hDBP complex. Reversible binding and covalent binding were monitored as with the azido-labeled samples. At an equivalent concentration of 25-OH-[26,27-^{3}H]D_3, 58% of the soluble vitamin was reversibly bound to hDBP prior to UV irradiation. The amount of reversibly bound vitamin decreased with UV irradiation as seen in Figure 2. In the presence of excess 25-OH-D_3, 95% of the tritiated compound was displaced from the protein, and the nonspecific binding was unchanged after exposure to UV light. There was a low detectable level of specific covalently bound 25-OH-[26,27-^{3}H]D_3 to hDBP, monitored by the filter assay, that increased with increasing exposure to UV light and reached a maximum of 0.9% of the reversibly bound sterol prior to irradiation. A fluorogram of an SDS gel of the UV-irradiated 25-OH-[26,27-^{3}H]D_3–hDBP samples also revealed low levels of specific covalently bound sterol after an 8-week exposure of the film to the gel. Calculations based on the parallel experiments of UV irradiation of Az-25-OH-[26,27-^{3}H]–hDBP and 25-OH-[26,27-^{3}H]D_3–hDBP indicate that at least 85% of the Az-25-OH-D_3 incorporation can be attributed to the azido component.

Saturating levels of hDBP were incubated with varying concentrations of Az-25-OH-[26,27-^{3}H]D_3, and the amount of reversible binding and covalent binding after 4 min of UV irradiation was measured in the samples (Table I). Both the reversible binding and covalent binding increased with increasing concentrations of azido label. The percent of the label that bound reversibly to hDBP varied from 25% to 29%. The percent of the reversibly bound label that became covalently bound to the protein remained fairly constant. In the presence of 25-OH-D_3, the covalent binding was reduced 70–80%. An average of 3% of the reversibly bound label became covalently

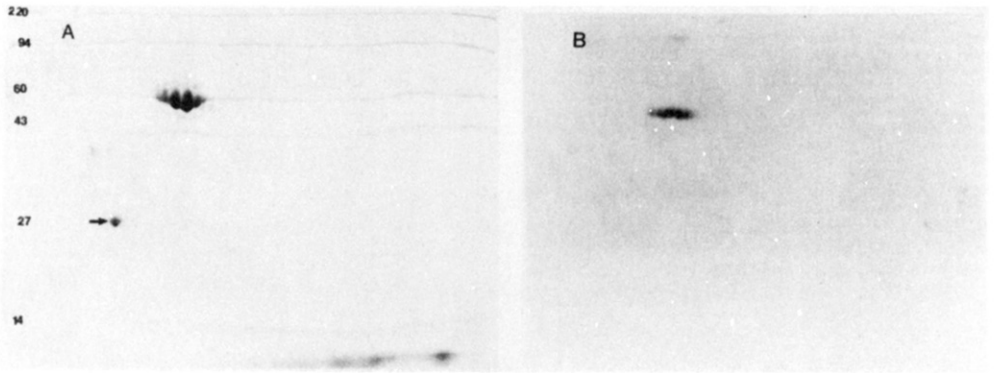

FIGURE 6: Two-dimensional electrophoresis of hDBP and fluorogram of Az-25-OH-[26,27-^{3}H]D_3-labeled hDBP. (A) A Coomassie blue stained two-dimensional electrophoresis gel of 5 mg of hDBP. The arrow indicates an internal standard, vitamin D dependent calcium binding protein, p*I* 4.2, M_r 27 000. (B) A fluorogram of a two-dimensional electrophoresis gel of covalently Az-25-OH-[26,27-^{3}H]D_3-labeled hDBP. hDBP (10 μg/mL) was incubated with Az-25-OH-[26,27-^{3}H]D_3 (2 nM) for 18 h at 0 °C in the dark and then UV irradiated for 3 min. A 150-μL aliquot was prepared for two-dimensional gel electrophoresis.

Table I: Reversible Binding and Covalent Binding of Az-25-OH-[26,27-^{3}H]D_3 to hDBP[a]

Az-25-OH-[26,27-^{3}H]D_3 (fmol)	reversibly bound[b] label (Br) (fmol)	covalently bound[c] label (Bc) (fmol)	Bc/Br[d] × 100
118	34	1.1	3.3
215	57	1.4	2.4
390	98	3.2	3.3

[a] hDBP (10 μg/mL) in PBS, pH 7.4, was incubated with Az-25-OH-[26,27-^{3}H]D_3 for 18 h at 0 °C in the dark in the absence or presence of excess 25-OH-D_3. Duplicate 250-μL samples were irradiated for 4 min with shortwave UV light. The Az-25-OH-[26,27-^{3}H]D_3 concentrations of 0.47, 0.86, and 1.56 nM are expressed as femtomoles in the 250-μL samples (1 fmol = 352 dpm). [b] Reversibly bound label was determined by HA assay for samples prior to UV irradiation. The data represent the specifically bound label expressed as femtomoles in 250 μL. [c] Covalently bound label was determined by filter assay for UV-irradiated samples. The data represent the specifically bound label expressed as femtomoles in 250 μL. [d] The percent of the reversibly bound label that covalently bound to hDBP with UV irradiation.

bound to the vitamin D binding site of hDBP. This represents an average of 0.8% of the soluble Az-25-OH-[26,27-^{3}H]D_3 in the assay.

Covalently Az-25-OH-[26,27-^{3}H]D_3-labeled hDBP, prepared from a 3-min UV-irradiated sample, was subjected to two-dimensional gel electrophoresis. A fluorogram of this gel is seen next to a Coomassie blue stained two-dimensional electrophoresis gel of hDBP in Figure 6. The photoaffinity labeling of hDBP, which can be easily visualized on a two-dimensional gel, shows incorporation into all isoelectric forms of the protein.

Covalent Binding of Azido Label to Human Plasma Proteins. The selectivity of the azido label was studied by monitoring the covalent binding of the azido label to proteins in human plasma. Frozen plasma was thawed and diluted 30-fold in PBS for a final DBP concentration of approximately 150 nM. The diluted plasma was incubated with 1.3 nM Az-25-OH-[26,27-^{3}H]D_3 in the absence and presence of 5 μM 25-OH-D_3. A parallel experiment containing an equivalent concentration of 25-OH-[26,27-^{3}H]D_3 was conducted as a control. The reversible binding assay showed 12% of the azido label and 54% of the 25-OH-[26,27-^{3}H]D_3 bound to protein. In the presence of excess 25-OH-D_3, the amount of reversibly bound tritium to protein is decreased 85% for Az-25-OH-[26,27-^{3}H]D_3 and 95% for 25-OH-[26,27-^{3}H]D_3. The reversible binding assay, which monitors tight binding, is a good indication of the amount of compound bound to DBP. After exposure to 3 min of UV irradiation, the covalent binding was determined by the filter assay. The filter assay showed that

Table II: Reversible Binding and Covalent Binding of Az-25-OH-[26,27-^{3}H]D_3 and 25-OH-[26,27-^{3}H]D_3 to Human Plasma Proteins

tritiated compd	concn (fmol)	25-OH-D_3 (mol)	reversibly bound[a] (fmol)	covalently bound[b] (fmol)
Az-25-OH-D_3	323	0	39	9.7
	325	1.25	5	8.6
25-OH-D_3	308	0	166	1.6
	298	1.25	9	1.5

[a] Reversibly bound label was determined by HA assay for samples prior to UV irradiation and expressed as femtomoles in 250 μL. [b] Covalently bound label was determined by filter assay for samples irradiated for 3 min with shortwave UV light and expressed as femtomoles in 250 μL.

3% of the Az-25-OH-[26,27-^{3}H]D_3 and 0.5% of the 25-OH-[26,27-^{3}H]D_3 were covalently bound to protein in either the absence or presence of excess 25-OH-D_3. The above data are summarized in Table II.

The amount of the azido label bound to DBP should be absent or reduced in the presence of excess 25-OH-D_3 even if the total covalent binding was the same. A fluorogram of the one-dimensional gel analysis showed that four and five proteins appear to covalently incorporate the azido label (Figure 7). Only a single band (arrow, Figure 7) was absent in the presence of excess 25-OH-D_3. DBP in plasma runs at this position because of the large amount of albumin in the sample. A fluorogram of the two-dimensional gel, which was Coomassie stained before treatment with ENHANCE and exposure to film, is seen in Figure 8 along with the stained gel. The only spots on the fluorogram that were absence when 25-OH-D_3 was present were the isomers of DBP (Figure 8a). The other three proteins that bound the azido label were identified by their position on a two-dimensional gel as b, albumin; c, α_1 antitrypsin; and d, apolipoprotein A-1. The majority of the azido label appears to be covalently bound to ablumin and apolipoprotein A-1 in a nonspecific manner.

Discussion

A tritiated photoactivatable analogue of 25-OH-D_3 was prepared for labeling of the vitamin D serum binding protein. Az-25-[26,27-^{3}H]D_3 was synthesized from 25-OH-[26,27-^{3}H]D_3, maintaining the high specific activity of the parent compound that allows detection of the label at low levels of incorporation. We have previously shown that Az-25-OH-D_3 is stable when stored in the dark at −70 °C, that it could be activated by shortwave UV light, and that it was a good

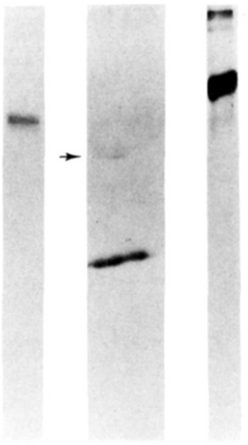

FIGURE 7: Fluorogram of Az-25-OH-[26,27-^{3}H]D_3-labeled human plasma. Human plasma was diluted 30-fold in PBS, pH 7.4, and incubated with 2 nM Az-25-OH-[26,27-^{3}H]D_3 for 18 h at 0 °C in the dark in the absence or presence of 5 μM 25-OH-D_3. Samples (250 μL) were irradiated for 3 min, and 140 μL was prepared for gel analyses. The fluorogram represnts an 8-week exposure of a 10% SDS–polyacrylamide gel to X-ray film. Lane 1 contains purified human DBP labeled with Az-25-OH-[26,27-^{3}H]D_3. Sample of Az-25-OH-[26,27-^{3}H]D_3 bound to human plasma proteins in the absence of 25-OH-D_3 is in lane 2 and in the absence of 25-OH-D_3 is in lane 3. Lane 4 contains [^{14}C]BSA as a molecular weight marker. The arrow marks a protein band that is present in lane 2 and absent in lane 3, which corresponds with the position of DBP in a overload plasma sample.

competitor for 25-OH-D_3 binding to human plasma (Kutner et al., 1986). The purpose of this study was to show that Az-25-OH-[26,27-^{3}H]D_3 binds at the 25-OH-D_3 binding site of hDBP and that the label will covalently bind to the protein when activated by UV light.

Az-25-OH-D_3 was shown to behave as a competitive inhibitor of 25-OH-D_3 binding to hDBP. Therefore, Az-25-OH-D_3 binds in the dark to the same site of hDBP as 25-OH-D_3. An apparent equilibrium inhibition constant (K_I) for Az-25-OH-D_3 binding to hDBP was found to be 30 nM. These findings are in close agreement with the competition study using human plasma (Kutner et al., 1986). Reversible binding studies of Az-25-OH-[26,27-^{3}H]D_3 to hDBP revealed that the azido compound had a low affinity for albumin, which was used as a carrier protein in all binding studies. An alternative carrier protein for albumin that does not interfere with 25-OH-D_3 binding could not be found. Therefore, low concentrations of azido label were employed in the Az-25-OH-[26,27-^{3}H]D_3 binding study, selecting for the higher affinity binder. A comparison of the reversible binding of the photolabel to 25-OH-D_3 binding to hDBP revealed an equivalent maximum number of binding sites and a 6-fold higher $K_{D,app}$ value for the photolabel. The direct binding study with the tritiated azido label predicted a higher affinity of the compound for hDBP than the competition studies. Although the need for a carrier protein prohibits obtaining an accurate equilibium constant, the binding studies clearly show that Az-25-OH-D_3 has high affinity for hDBP and binds at the 25-OH-D_3 binding site.

The Az-25-OH-[26,27-^{3}H]D_3 label was clearly shown to bind covalently to hDBP when exposed to UV light. Both assays used to monitor the covalent incorporation of label into the protein showed increasing amounts of tritium associated with the protein with increasing exposure to UV light. The optimal irradiation time was found to be between 3 and 5 min, at which point 4–6% of the reversibly bound label appeared to be covalently bound. A parallel control experiment using 25-OH-[26,27-^{3}H]D_3 showed a low level of covalent binding of the sterol to the protein. The amount of covalent binding of the azido compound was 5–6 times higher than the parent compound, indicating that approximately 85% of the covalent binding is through the activated azido group.

Evidence for attachment of the photolabel at the vitamin D binding site was obtained by preparing samples of Az-25-OH-[26,27-^{3}H]D_3 hDBP in the absence and presence of excess 25-OH-D_3. Saturating levels of hDBP were used in these experiments to maximize the amount of azido label bound and to eliminate the need for a carrier protein. The amount of 25-OH-D_3 added was in excess of the amount of hDBP so that all hDBP sites would be filled. The reversible binding data showed that 21% of the tritiated azido label was bound to hDBP after the overnight incubation in the dark and that 94% of the label was displaced from the protein when 25-OH-D_3 was present. An average of 3% of the reversibly bound label became covalently bound to the protein at the 25-OH-D_3 binding site.

Az-25-OH-[26,27-^{3}H]D_3 was used to label human plasma proteins. The azido label was incubated with human plasma in the absence and presence of 25-OH-D_3. A comparison of the labeled proteins in the two samples can then be used to identify the highest affinity binders of the parent compound.

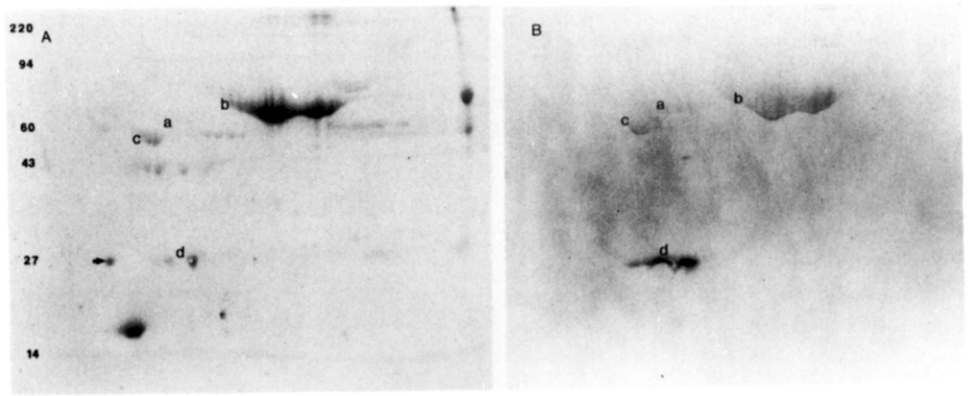

FIGURE 8: Two-dimensional electrophoresis of human plasma and a fluorogram of Az-25-OH-[26,27-^{3}H]D_3-labeled human plasma. Human plasma was diluted 30-fold in PBS, pH 7.4, and incubated with 2 nM Az-25-OH-[26,27-^{3}H]D_3 for 18 h at 0 °C in the dark. Samples (250 μL) were irradiated for 3 min, and 140 μL was prepared for two-dimensional gel electrophoresis. (A) The Coomassie blue stained two-dimensional electrophoresis gel of Az-25-OH-[26,27-^{3}H]D_3-labeled plasma. The arrow indicates an internal standard, vitamin D dependent calcium binding protein, pI 4.2, M_r 27 000. (B) A fluorogram of the two-dimensional electrophoresis gel, representing a 7-week exposure to X-ray film. The spots present on the fluorogram were identified by their position on the two-dimensional gel as (a) DBP, (b) albumin, (c) α_1 antitrypsin, and (d) apolipoprotein A-1.

DBP, which is the major plasma binding protein for 25-OH-D_3 (Silver & Fainaru, 1979), was the only protein that was labeled in the absence of 25-OH-D_3 and unlabeled in the presence of 25-OH-D_3. A high amount of nonspecific labeling was expected since it was not possible to remove the free label prior to UV irradiation. The reversible binding assay indicated that only 12% of the soluble azido label was bound to DBP. Therefore, 88% of the label was probably weakly associated with other proteins and lipoproteins in the plasma. A plasma protein could become nonspecifically labeled for any of the following reasons: (1) it was weakly associated with the azido label; (2) it was in very high concentration in plasma; or (3) it contained a good acceptor site for the free activated label. Three other plasma proteins covalently bound the azido label: albumin, apolipoprotien A-1, and α_1 antitrypsin. These proteins appear to be equivalently labeled in the absence or presence of 25-OH-D_3, indicating nonspecific binding. The majority of the affinity label was bound to albumin and apolipoprotein A-1. The Az-25-OH-D_3 labeling of these proteins may be due to their abundance in plasma or may be reflecting their interaction with vitamin D compounds. Albumin has been shown to be a secondary plasma carrier protein for 1,25-$(OH)_2$-D_3, especially when DBP levels are decreased or sterol levels are increased (Bikle et al., 1985; Silver & Fainaru, 1979). Lipoproteins appear to be particularly important in all transport and hepatic uptake of vitamin D (Thompson et al., 1969; Ziv et al., 1985). Among the lipoproteins, high-density lipoproteins (HDL) were considered to have the highest affinity for vitamin D (Silver & Fainaru, 1979). Apolipoprotein A-1 is the major protein component of HDL. It was sugested that vitamin D is associated with the surface of the lipoprotein (Ziv et al., 1985), which would put the sterol in the vicinity of apolipoprotein A-1. The Az-25-OH-D_3 compound may prove to be a useful tool for studies with these secondary plasma carrier proteins for vitamin D metabolites.

The high specific activity of the Az-25-OH-[26,27-^{3}H]D_3 allows for detection of the label into protein even at low levels of incorporation. Furthermore, the fluorograms presented here show clear identification of proteins on one-dimensional or two-dimensional gel electrophoresis.

An obvious goal is to identify the vitamin D binding region of hDBP. Such an investigation is currently under way in this laboratory. This will also confirm whether the azido label is associated with a defined region of the protein or randomly dispersed in the structure. The vitamin D binding domain of DBP is of great interest now that the amino acid sequence of the protein is available (Cooke & David, 1985; Schoenten et al., 1986; Yang et al., 1985). The amino acid sequence of hDBP showed a three-domain structure with strong homology to albumin (Cooke & David, 1985; Schoenten et al., 1986; Yang et al., 1985). The identification of the vitamin D binding domain would begin the designation of function to particular domain of the protein.

Registry No. 25-OH-D_3, 19356-17-3; Az-25-OH-D_3, 108345-00-2; Az-25-OH-[26,27-^{3}H]D_3, 108344-99-6; 25-OH-[26,27-^{3}H]D_3, 71595-10-3.

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