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Photoaffinity Labeling of the Rat Plasma Vitamin D Binding Protein with [26,27-^{3}H]-25-Hydroxyvitamin D_3 3β-[*N*-(4-Azido-2-nitrophenyl)glycinate]†

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ABSTRACT: It is well recognized that the vitamin D binding protein (DBP) is important for the transport of vitamin D, 25-hydroxyvitamin D (25-OH-D), and its metabolites. In an attempt to better understand the molecular-binding properties of this ubiquitous protein, we designed and synthesized a photoaffinity analogue of 25-OH-D_3 and its radiolabeled counterpart. This analogue, 25-hydroxyvitamin D_3 3β-[*N*-(4-azido-2-nitrophenyl)glycinate] (25-OH-D_3-ANG), was recognized by the rat DBP and was about 10 times less active than 25-OH-D_3 in terms of binding. Incubation of [^{3}H]25-OH-D_3 or [^{3}H]25-OH-D_3-ANG with rat DBP revealed that both compounds were specifically bound to a protein with a sedimentation coefficient of 4.1 S. Each was displaced with a 500-fold excess of 25-OH-D_3. When [^{3}H]25-OH-D_3-ANG was exposed to UV radiation in the presence of rat DBP followed by the addition of a 500-fold excess of 25-OH-D_3, there was no displacement of tritium from the 4.1S peak. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis autoradiographic analysis of [^{3}H]25-OH-D_3-ANG exposed to UV radiation in the presence of rat DBP followed by the addition of a 500-fold excess of 25-OH-D_3 revealed one major band with a molecular weight of 52 000. These data provide strong evidence that [^{3}H]25-OH-D_3-ANG was covalently linked to the rat DBP. This photoaffinity probe should provide a valuable tool for the analysis of the binding site on this transport protein.

The group-specific component (Gc) in blood occurs in more than 300 distinct populations throughout the world. Its regular frequency of occurrence and well-characterized polymorphic pattern in electrophoresis identify it as a genetic marker. However, no specific biologic role was assigned to it until 1975, when the discovery was made accidentally that Gc recognizes and binds to vitamin D and its metabolites in a very specific manner (Daiger et al., 1975; Daiger, 1979; Thomas et al., 1959; Haddad & Walgate, 1976a,b; Van Baelen et al., 1978). It is now firmly established that Gc is the protein responsible for the transport of vitamin D and its metabolites from the circulation to target tissues. DBP consists of a single polypeptide chain of molecular weight 51 000–58 000 (Haddad & Walgate, 1976a,b). It contains a very small percentage of carbohydrates containing sialic acid that are sensitive to neuraminidase (Svasti & Bowman, 1978). Amino acid compositions of human and rat DBP have been determined and are comparable (Bouillon et al., 1976, 1978). Furthermore, Svasti et al. (1979) determined the amino acid sequence of the amino and carboxy termini of the protein from the tryptic hydrolysate of ^{14}C-carboxymethylated DBP, and Cooke (1986) has recently determined the complete amino acid sequence of the rat DBP from a cloned cDNA. Photoaffinity-labeling techniques have been used extensively to localize and study membrane-bound and cytoplasmic receptors for several steroid hormones among various other macromolecules (Bayley & Knowles, 1977; Das & Fox, 1979). Recently, we reported on the synthesis of a photoaffinity probe for 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2$-D_3] and presented evidence that it could be covalently attached to the binding site of the chick intestinal cytosolic 1,25-$(OH)_2$-D_3 receptor (Ray et al., 1985a,b). We now report on the synthesis of a photoaffinity-labeled derivative of 25-OH-D_3 and its radiolabeled

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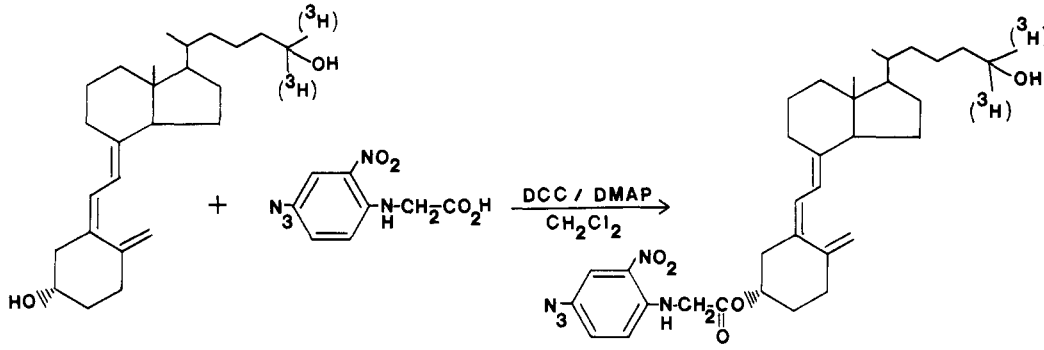

FIGURE 1: Synthetic scheme for 25-hydroxyvitamin D_3 3β-[*N*-(4-azido-2-nitrophenyl)glycinate] (25-OH-D_3-ANG) or [26(27)-*methyl*-^{3}H]-25-hydroxyvitamin D_3 3β-[*N*-(4-azido-2-nitrophenyl)glycinate] ([^{3}H]25-OH-D_3-ANG).

counterpart. This analogue, 25-hydroxyvitamin D_3 3β-[*N*-(4-azido-2-nitrophenyl)glycinate] (25-OH-D_3-ANG) has a photolabile azidonitrophenyl group attached to the 3-hydroxyl group of the 25-OH-D_3 skeleton via a glycine side chain. We have used 25-OH-D_3-ANG and [26(27)-*methyl*-^{3}H]-25-hydroxyvitamin D_3 3β-[*N*-(4-azido-2-nitrophenyl)glycinate] ([^{3}H]25-OH-D_3-ANG) to analyze and label DBP from vitamin D deficient rat plasma.

Experimental Procedures

Materials. 25-OH-D_3 was a generous gift from Dr. John Babcock of Upjohn Co., Kalamazoo, MI. [26(27)-*methyl*-^{3}H]25-OH-D_3 (sp act. 20.6 Ci/mmol) and the ^{14}C-methylated protein mixture of molecular weight determination containing ^{14}C-methylated myosin (200 000), ^{14}C-methylated phosphorylase *b* (92 500), ^{14}C-methylated bovine serum albumin (69 000), ^{14}C-methylated ovalbumin (46 000), and ^{14}C-methylated carbonic anhydrase (30 000) were purchased from Amersham Corp., Arlington Heights, IL. 4-Fluoro-2-nitrophenyl azide was obtained from Pierce Chemical Corp., Rockford, IL. All other chemicals and solvents were analytically pure, were available commercially, and were used without further purification. Anhydrous dichloromethane was obtained by distilling commercial dichloromethane from sodium and benzophenone ketyl and storing it over 4-Å molecular sieves. UV absorption spectra were obtained on a Perkin-Elmer 552A UV–vis spectrophotometer. IR spectra were taken in a Perkin-Elmer infrared spectrophotometer. NMR spectra were taken in a Bruker NMR spectrometer, 250 MHz, with $CDCl_3$ as solvent and tetramethylsilane as internal standard. Irradiation experiments were performed with a Hanovia medium-pressure mercury arc lamp (MacLaughlin et al., 1982). Weanling male Holtzman rats (Holtzman Co., Madison, WI) maintained on a vitamin D deficient diet containing 0.47% calcium and 0.4% phosphorus for 4 weeks were sacrificed, and the blood, which contained an undetectable amount of 25-OH-D_3, was collected for serum. The assay buffer (400 mL) was prepared by adding 400 mg of purified bovine serum albumin (BSA) (Pierce) and 0.1 g of sodium azide to 20 mL of barbital acetate solution (containing 0.19 g of sodium acetate and 0.32 g of sodium barbital in deionized water) and bringing up the pH to 8.6. Adsorbent charcoal was prepared by mixing 0.3 g of dextran T70 and 3 g of Norit GSX in the assay buffer without BSA. Insta-Gel scintillation cocktail was obtained from Packard Instruments Co., Chicago, IL. Enlightning autoradiography enhancer was purchased from New England Nuclear, Boston, MA. Exposure to direct light was strictly avoided.

Synthesis of 25-Hydroxyvitamin D_3 3β-[N-(4-Azido-2-nitrophenyl)glycinate] (25-OH-D_3-ANG). The title compound was prepared according to the reaction shown in Figure 1. A solution of 25-OH-D_3 (1.12 mg), dicyclohexylcarbodiimide (DCC, 1.2 mg), 4-*N*,*N*-dimethylaminopyridine (DMAP, 0.7 mg), and *N*-(4-azido-2-nitrophenyl)glycine (ANP-glycine, 2 mg) (Fleet et al., 1972) in 1.5 mL of anhydrous dichloromethane was stirred under argon in the dark for 18 h. The resulting red solution was concentrated under nitrogen and purified by preparative TLC using 2:1 hexane–ethyl acetate as eluant. The band having an R_f of approximately 0.8 was isolated as an orange solid in 73% yield: UV (ethanol) 259–260 (strong) and 244–246 nm (weak and broad); IR (solution in chloroform) 1745 (carbonyl), 2130 (azide), and 3300–3600 cm^{-1} (br, NH and OH); 250-MHz ^{1}H NMR ($CDCl_3$) δ 0.54 (s, 3 H, Me-18), 0.94 (d, 3 H, J = 6.08 Hz, Me-21), 1.22 (s, 6 H, Me_2-26,27), 4.09 (d, 2 H, J = 5.42 Hz, CH_2-CO), 4.86 and 5.08 (br s, 2 H, H-19), 5.12 (m, 1 H, H-3), 6.01 and 6.19 (AB q, 2 H, J = 11.1 Hz, H-6,7), 6.71 (d, 1 H, H-aromatic), 7.12 and 7.16 (dd, 1 H, NH), 7.91 (narrow d, 1 H, H-aromatic), and 8.4 (m, 1 H, H-aromatic).

Synthesis of [26(27)-methyl-^{3}H]25-OH-D_3 3β-[N-(4-Azido-2-nitrophenyl)glycinate] ([^{3}H]25-OH-D_3-ANG). [26(27)-*methyl*-^{3}H]25-OH-D_3 (5 μCi, sp act. 20.6 Ci/mmol) was treated in an identical fashion as above (Figure 1). The adduct was purified by preparative HPLC (silica column, 1% 2-propanol–hexane, 3 mL/min). The peak corresponding to the authentic cold sample of 25-OH-D_3-ANG was isolated. The yield of [3H]25-OH-D_3-ANG was 2.7 μCi (53%).

Competitive Binding Assays for 25-OH-D_3 and 25-OH-D_3-ANG. Various concentrations of 25-OH-D_3 (0.06–4.0 pmol) or 25-OH-D_3-ANG (0.2–161.6 pmol), dissolved in 50 μL of ethanol, were added to tubes in triplicate. [^{3}H]25-OH-D_3 (1800 cpm, sp act. 20.6 Ci/mmol) dissolved in 20 μL of ethanol was added to each tube (Schacter et al., 1961). A total of 0.5 mL of vitamin D deficient rat serum (diluted 1:16 000 with the assay buffer) was added to each tube, and all the tubes were incubated in the dark at 4 °C for 18 h, followed by addition of 100 μL of the charcoal suspension to each tube and incubation at 25 °C for 15 min. The tubes were centrifuged (3000 rpm, 4 °C, 20 min). Supernatant from each tube was mixed with 10 mL of Insta-Gel and counted for radioactivity in a β-liquid scintillation counter.

Sucrose Density Gradient Sedimentation Analysis with DBP, [^{3}H]25-OH-D_3, and [^{3}H]25-OH-D_3-ANG. Six tubes, each containing 300 μL of vitamin D deficient rat serum (diluted 1:10 with the assay buffer), were incubated (0 °C, 3 h) with (a) [^{3}H]25-OH-D_3 (100 000 cpm, sp act. 20.6 Ci/mmol) in 20 μL of ethanol, (b) [^{3}H]25-OH-D_3 (100 000 cpm, sp act. 20.6 Ci/mmol) and 1 μg of 25-OH-D_3 in 20 μL of ethanol, (c, e, and f) [^{3}H]25-OH-D_3-ANG in each (100 000 cpm, sp act. 20.6 Ci/mmol) in 20 μL of ethanol, and (d) [^{3}H]25-OH-D_3-ANG (100 000 cpm, sp act. 20.6 Ci/mmol) and 1 μg of 25-OH-D_3 in 20 μL of ethanol. After the incu-

bation, samples e and f were irradiated for 15 min at 0 °C with a Hanovia medium-pressure mercury arc lamp. The lamp was held approximately 10 cm above the solutions, which were magnetically stirred. Following the irradiation, all the samples except sample f were incubated with 100 μL of the ice-cold charcoal suspension for 15 min at 0 °C followed by centrifugation at 3000 rpm at 4 °C for 10 min. Sample f, in the mean time, was incubated with 25-OH-D_3 (1 μg in 20 μL of ethanol) at 0 °C for 3 h, treated with charcoal (100 μL, 0 °C, 15 min), and centrifuged at 3000 rpm (4 °C, 10 min). A total of 150 μL of supernatant from each sample was saved for electrophoresis (see next section). The remainder of the supernatant (200 μL) from each sample was gently layered at the top of a 4–20% sucrose density gradient made with 2.4 mL each of 4% and 20% sucrose solutions with the assay buffer. The gradients were then centrifuged at 49000*g* for 16 h at 4 °C. After the centrifugation, each gradient was fractionated (5 drops each) from the bottom directly into scintillation vials. Each fraction was mixed with 4 mL of Insta-Gel and counted for radioactivity. In a separate control experiment, 300 μL of vitamin D deficient serum was aliquoted into two tubes. [^{3}H]25-OH-D_3 (100000 cpm, sp act. 20.6 Ci/mmol) was added to each tube. 25-OH-D_3 (1 μg) was added to one of the tubes. Both tubes were incubated at 0 °C for 3 h. Both samples were then irradiated for 15 min followed by treatment with charcoal. Supernatants from both the solutions were layered on 4–20% sucrose density gradients and treated the same way as in the previous cases. ^{14}C-Methylated bovine serum albumin was used as the molecular weight standard.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE) Analysis with DBP and [^{3}H]25-OH-D_3-ANG. Aliquots of 150 μL from samples d, e, and f from the above-mentioned experiment were mixed with 50 μL of sample buffer made by dissolving 1 g of SDS in 24.75% of 1 M tris(hydroxymethyl)aminomethane (Tris) (pH 6.8), 50% glycerol, 25% β-mercaptoethanol, and 0.25% bromophenol blue. All the samples were heated in boiling water for 2 min and applied to lanes on a 7% polyacrylamide gel. On a separate lane was applied a sample containing the ^{14}C-methylated protein mixture (Amersham) in the electrophoresis sample buffer (Laemmli, 1970). The gel was run at a constant current of 30 mA. After the electrophoresis, the gel was immersed in Enlightning autoradiography enhancer (New England Nuclear). The solution was agitated gently for 15 min and then dried on a slab-gel drier. Autoradiography was performed by exposing the dried gel on Kodak XAR-5 film at −70 °C for 10 days.

Results and Discussion

We hereby report, for the first time, the chemical synthesis of a photoaffinity-labeled analogue of 25-OH-D_3 and its radiolabeled counterpart. We chose to derivatize the 3β-hydroxyl group of the 25-OH-D_3 molecule because structure–function studies have shown that this position, on derivatization, is least likely to cause serious alteration in its binding properties (Belsey et al., 1974). The key step in the synthesis of 25-OH-D_3-ANG and its tritiated derivative (Figure 1) involved DCC coupling of 25-OH-D_3 or [^{3}H]25-OH-D_3 with a glycine derivative in which the amino group is attached to the photolabile 4-azido-2-nitrophenyl group. Physical constants (NMR, IR, and UV spectral data) of 25-OH-D_3-ANG were commensurate with its structure.

Results of the competitive binding assays are displayed in Figure 2; 3.3 pmol of 25-OH-D_3-ANG and 0.34 pmol of 25-OH-D_3, respectively, were needed to displace 50% maximally

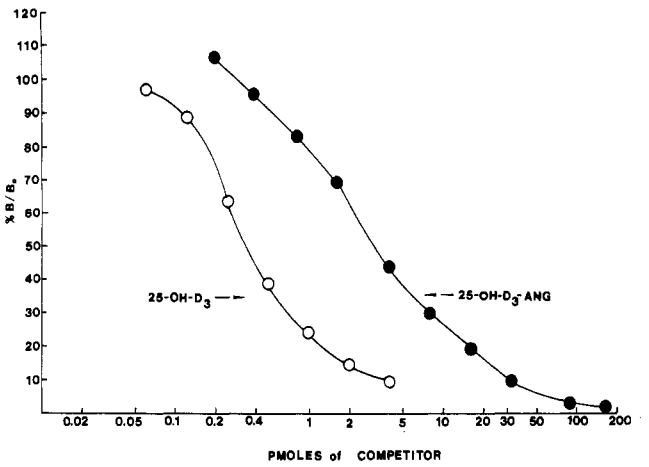

FIGURE 2: Competitive binding assays for rat plasma vitamin D binding protein. Various concentrations of 25-OH-D_3 (○) and 25-OH-D_3-ANG (●) were incubated with [^{3}H]25-OH-D_3 and DBP in the assay buffer (see text). Following incubation, unbound sterols were removed by treatment with charcoal, and supernatants were counted for radioactivity.

bound [^{3}H]25-OH-D_3 from the specific binding site of DBP. Thus, 25-OH-D_3-ANG was approximately 10 times less active than the natural substrate, i.e., 25-OH-D_3. This result confirmed our initial assessment that the derivatization at the 3β-position would not seriously hamper the specific recognition of this analogue by DBP.

Sucrose density gradient sedimentation profiles are shown in Figure 3. A single radioactive peak, having a sedimentation coefficient of 4.1 S, which was displaceable by a 500-fold excess of 25-OH-D_3, was observed in the case of [^{3}H]25-OH-D_3 (Figure 3A). This indicated a specific binding of [^{3}H]25-OH-D_3 to DBP. A very similar result was observed for [^{3}H]25-OH-D_3-ANG (Figure 3B). Displacement of most of the radioactivity by pulsing with a 500-fold excess of 25-OH-D_3 confirmed specific recognition and binding of [^{3}H]-25-OH-D_3-ANG by DBP (Figure 3B). UV irradiation of rat serum preincubated with [^{3}H]25-OH-D_3-ANG produced a radioactive peak having a sedimentation coefficient similar to that for [^{3}H]25-OH-D_3 (Figure 3C), indicating covalent binding of [^{3}H]25-OH-D_3-ANG to DBP. Covalent binding of [^{3}H]25-OH-D_3-ANG was further confirmed when post-pulsing with a 500-fold excess of 25-OH-D_3 failed to displace the radioactive peak from a sample that was UV-irradiated after incubation with [^{3}H]25-OH-D_3-ANG (Figure 3C).

To be certain that [^{3}H]25-OH-D_3 could not be covalently attached by the irradiation, DBP was irradiated after incubation with either [^{3}H]25-OH-D_3 (10000 cpm) alone or with [^{3}H]25-OH-D_3 and 1 μg of 25-OH-D_3. Sucrose density gradient analysis of these samples (results not shown) revealed a 4.1S radioactive peak that was completely displaced by an excess of 25-OH-D_3. These results proved that [^{3}H]25-OH-D_3 could not be directly photochemically attached to the DBP without the photolabile 4-azido-2-nitrophenyl group.

An autoradiographic analysis of the SDS–PAGE for the UV-irradiated DBP sample that was preincubated with [^{3}H]25-OH-D_3-ANG revealed a single intense band along with other bands of much less intensity (Figure 4, lane 2). This labeled band corresponded to a molecular weight of approximately 52000, in comparison with bands corresponding to standard molecular weight marker proteins (shown by arrows in Figure 4). This value for the molecular weight of DBP matched very well with the assigned molecular weight of 51000–55000 (Haddad & Walgate, 1976a,b). An identical fingerprint profile was obtained from a sample of DBP, pulsed

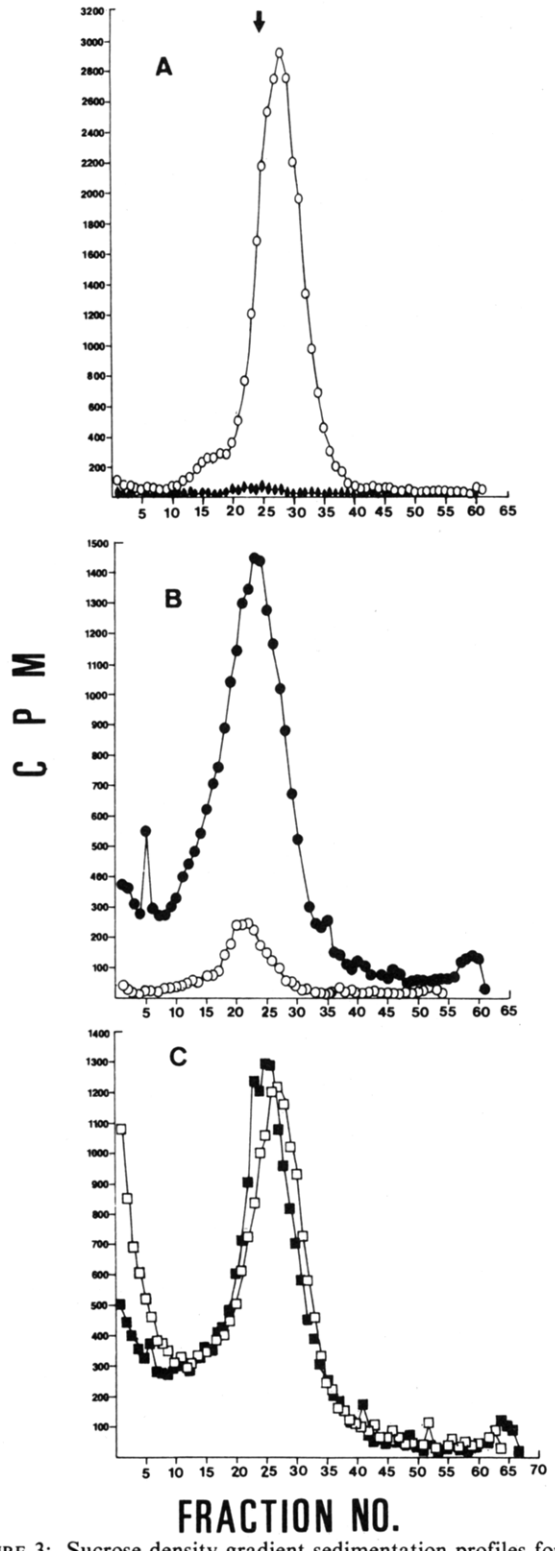

FIGURE 3: Sucrose density gradient sedimentation profiles for rat plasma DBP incubated with [^{3}H]25-OH-D$_3$ or [^{3}H]25-OH-D$_3$-ANG. For all the samples, unbound sterols were removed by treatment with charcoal, and an aliquot from each supernatant was run through a 4–20% sucrose density gradient at 49000*g*. The gradients were then fractionated from the bottom of the tubes, with the radioactivity in each 5-drop fraction being determined by β-liquid scintillation counting. (A) A sample of DBP was incubated with [^{3}H]25-OH-D$_3$ alone (○) or in the presence of a 500-fold excess of 25-OH-D$_3$ (●). (B) A sample of DBP was incubated with [^{3}H]25-OH-D$_3$-ANG alone in the dark (●) or in the presence of an excess of 25-OH-D$_3$ (○). (C) A sample of DBP, after incubation with [^{3}H]25-OH-D$_3$-ANG, was UV-irradiated (□) or treated with an excess of 25-OH-D$_3$ following UV irradiation (■). The arrow in the top of this figure indicates the position of the radioactive peak corresponding to the ^{14}C-methylated bovine serum albumin marker (4.4 S).

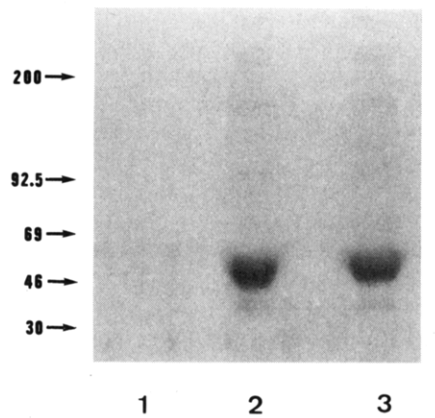

FIGURE 4: Autoradiogram of a sodium dodecyl sulfate–polyacrylamide gel of rat plasma DBP photoaffinity-labeled with [^{3}H]25-OH-D$_3$-ANG. Aliquots of 150 μL each of DBP + [^{3}H]25-OH-D$_3$-ANG + a 500-fold excess of 25-OH-D$_3$ (lane 1), DBP + [^{3}H]25-OH-D$_3$-ANG + UV irradiation (lane 2), and DBP + [^{3}H]25-OH-D$_3$-ANG + UV irradiation + a 500-fold excess of 25-OH-D$_3$ (lane 3) were denatured (see text) and applied to the gel. A mixture of ^{14}C-methylated standard molecular weight proteins (Amersham Corp.) was applied to a separate lane (not shown). After the electrophoresis, the gel was briefly immersed in autoradiography enhancer, dried, and autoradiographed at −70 °C for 10 days (see text). Bands corresponding to standard molecular weights (×10^3) are designated by arrows.

with an excess of 25-OH-D$_3$ following irradiation (lane 3). No labeling was observed when DBP was incubated with [^{3}H]-25-OH-D$_3$-ANG and an excess of 25-OH-D$_3$ (lane 1), ruling out the possibility of a nonspecific and non-UV-related binding. Combining all these results, we have come to the conclusion that [^{3}H]25-OH-D$_3$-ANG produced a highly reactive nitrene intermediate upon irradiation, which, in turn, became covalently and preferentially linked to DBP.

We hereby report a relatively simple yet efficient synthesis of a photoaffinity-labeled derivative of 25-OH-D$_3$ and its radioactive counterpart. The lack of any previous attempt to determine the structure of the site where the DBP molecule binds to vitamin D and other vitamin D metabolites is probably due to the unavailability of a proper probe for this macromolecule. The availability of a photoaffinity-labeled derivative of 25-OH-D$_3$ and its radiolabeled counterpart described in this paper in conjunction with the knowledge of the full-length primary structure of the rat DBP (Cooke, 1986) could prove to be an important tool in attaining this goal.

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Registry No. 25-OH-D$_3$, 19356-17-3; ANP-glycine, 38873-75-5; 25-OH-D$_3$-ANG, 101396-04-7; [26(27)-*methyl*-^{3}H]25-OH-D$_3$, 103498-85-7; [^{3}H]25-OH-D$_3$-ANG, 103498-86-8.

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Articles

Cholesterol-Dependent Modification of Microsomal Dynamics and UDPglucuronyltransferase Kinetics†

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ABSTRACT: The effect of both in vitro incorporation and removal of cholesterol in guinea pig liver microsomes on the lipid composition, dynamic properties of the membrane, and kinetic constants of UDPglucuronyltransferase was studied. No significant changes either in the fatty acid composition or in the distribution of phospholipid classes were observed upon cholesterol incorporation and removal. Lateral and rotational mobility measured by the efficiency of pyrene excimer formation and fluorescence of 1,6-diphenylhexatriene decreased with cholesterol incorporation and increased in parallel to cholesterol removal. These changes were associated with alterations in the kinetic properties of UDPglucuronyltransferase. Whereas V_{max} increased, the K_m of the different steps of the reaction decreased with cholesterol incorporation. The negative homotropic effect and apparent cooperativity of UDP–glucuronic acid decreased when cholesterol was incorporated and increased after cholesterol removal. Moreover, the UDP–*N*-acetylglucosamine-dependent activation of the enzyme decreased in correlation with an increase of cholesterol concentration in microsomes. It has been demonstrated that both the shift of the non-Michaelian kinetics of the enzyme to Michaelian and the decrease of the UDP–*N*-acetylglucosamine-dependent activation of the enzyme are evoked by a change of the physical state of the UDPglucuronyltransferase milieu from a gel phase to a liquid-crystalline phase. Therefore, we must admit that cholesterol incorporation in the microsomes while producing an increased packing of the bulk lipids would also cause the separation of more fluid phospholipids, which increase the proportion of molecules in the liquid-crystalline state within the enzyme environment.

The kinetic properties of several membrane-bound enzymes including liver UDPglucuronyltransferase depend on the composition and physical state of the lipid bilayer phase (Strobel et al., 1970; Zakim & Vessey, 1976). The lipid modulation of this specific enzyme has been interpreted according to the compartmentation theory (Berry & Hallinan, 1976) as an effect on the substrate transport to the deeply located catalytic sites. This theory has been elaborated farther by considering that two enzyme forms, buried and exposed, contribute to the characteristic kinetic properties of the reaction (Onah et al., 1982; Cummings et al., 1984). However, relevant information has been also gathered by Zakim et al. (Zakim & Vessey, 1975, 1978, 1982) to suggest a direct effect on the enzyme molecule.

Since cholesterol is an important modulator of membrane physical properties condensing the lipid bilayer (De Kruijff et al., 1973) above the transition temperature, we investigated the effect of this lipid on the kinetic properties of UDPglucuronyltransferase. This study is important for understanding better not only the mechanism of interaction between lipid bialyers and associated enzymes but also the modulation of the UDPglucuronyltransferase reaction. Besides, it broadens our knowledge on the way cholesterol interacts with membranes and enzymes.

In a previous work (Castuma et al., 1986) we showed that an enhancement of dietary cholesterol in guinea pigs increased the microsomal cholesterol content by decreasing membrane "fluidity" and modifying the kinetic properties of the enzyme. Not only the K_ms and V_m were modified, but also the non-

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