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Synthesis of 25-Hydroxyvitamin D_3 3β-3′-[*N*-(4-Azido-2-nitrophenyl)amino]propyl Ether, A Second-Generation Photoaffinity Analogue of 25-Hydroxyvitamin D_3: Photoaffinity Labeling of Rat Serum Vitamin D Binding Protein†

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ABSTRACT: Vulnerability of 25-hydroxy-[26,27-^{3}H]vitamin D_3 3β-*N*-(4-azido-2-nitrophenyl)glycinate, a photoaffinity analogue of 25-hydroxyvitamin D_3 (25-OH-D_3) (Ray et al., 1986) toward standard conditions of carboxymethylatioin promoted us to synthesize 25-hydroxyvitamin D_3 3β-3′-[*N*-(4-azido-2-nitrophenyl)amino]propyl ether (25-ANE), a hydrolytically stable photoaffinity analogue of 25-OH-D_3, and 25-hydroxyvitamin D_3 3β-3′-[*N*-(4-azido-2-nitro-[3,5-^{3}H]phenyl)amino]propyl ether (^{3}H-25-ANE), the radiolabeled counterpart of 25-ANE. Competitive binding assays of 25-OH-D_3 and 25-ANE with rat serum demonstrated that 25-ANE competes for the 25-OH-D_3 binding site in rat serum vitamin D binding protein (rDBP). On the other hand, UV exposure of a sample of purified rat DBP (rDBP), preincubated in the dark with ^{3}H-25-ANE, covalently labeled the protein. However, very little covalent labeling was observed in the absence of UV light or in the presence of a large excess of 25-OH-D_3. These results provide strong evidence for the covalent labeling of the 25-OH-D_3 binding site in rDBP by ^{3}H-25-ANE.

It is well established that vitamin D binding protein (DBP), a major serum constituent, transports vitamin D and its metabolites to target organs and tissues leading to the observed calciotropic properties of the vitamin D hormone (DeLuca, 1979; Cooke & Haddad, 1989). DBP binds to metabolites of vitamin D with high affinities, and one of the highest affinities is toward 25-hydroxyvitamin D_3 (25-OH-D_3) (Francheschi et al., 1981; Daiger et al., 1975; Haddad & Walgate, 1976).

Recently other functions of DBP have been recognized. DBP binds and sequesters monomers of actin with high affinity (Van Baelen et al., 1980; Haddad, 1982). DBP has also been found to be associated with unsaturated fatty acids (Williams et al., 1988; D. Z. Xiang and R. Bouillon, unpublished results) as well as with various cell types including B- and T-lymphocytes and the cytotrophoblasts of placenta (Petrini et al., 1983, 1985). The physiologic significance of the observed multifunctional properties of DBP remains unknown. On the other hand, the primary amino acid structures of human and rat serum DBPs have been determined recently (Yang et al., 1985; Cooke & David, 1985; Schoentgen et al., 1986; Cooke, 1986).

Photoaffinity labeling has been a very important biochemical tool for probing ligand-binding sites of steroid hormone binding

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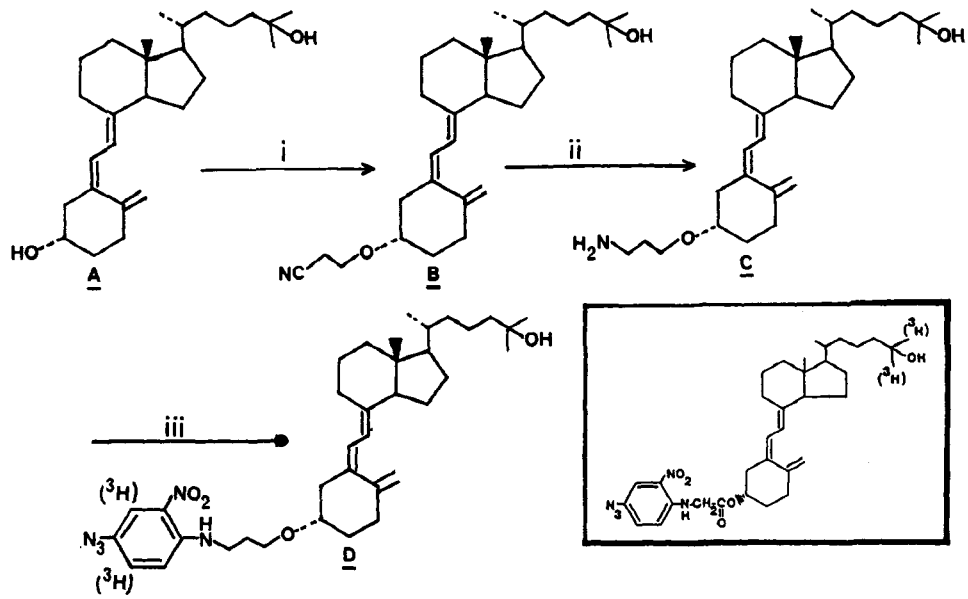

FIGURE 1: Scheme for the synthesis of 25-hydroxyvitamin D_3 3β-3′-[*N*-(4-azido-2-nitrophenyl)amino]propyl ether (25-ANE) and 25-hydroxyvitamin D_3 3β-3′-[*N*-(4-azido-2-nitro-[3,5-^{3}H]phenyl)amino]propyl ether (^{3}H-25-ANE). (i) CH_2=CH–CN/Triton B/*t*-BuOH–CH_3CN, 4 °C. (ii) $LiAlH_4$–$AlCl_3$ (1:1)/ether, 25 °C. (iii) 4-Fluoro-2-nitrophenyl azide (4-fluoro-2-nitro-[3,5-^{3}H]phenyl azide)/Et_3N/DMSO, 60 °C. Inset: 25-hydroxyvitamin D_3 3β-*N*-(4-azido-2-nitro-[3,5-^{3}H]phenyl)glycinate (^{3}H-25-ANG).

proteins, among various other biomolecules (Sweet & Murdock, 1987; Bayley & Knowles, 1977). Previously we described the synthesis of 25-hydroxy-[26,27-^{3}H]vitamin D_3 3β-*N*-(4-azido-2-nitrophenyl)glycinate, a photoaffinity analogue of 25-OH-D_3, and its successful application in photoaffinity labeling of rat serum vitamin D binding protein (rDBP) in crude rat serum (Ray et al., 1986). Link et al. have developed yet another photoaffinity analogue of 25-OH-D_3 and reported photoaffinity labeling of human serum DBP (hDBP) with this analogue (Link et al., 1987).

In this communication, we report the synthesis of 25-hydroxyvitamin D_3 3β-3′-[*N*-(4-azido-2-nitrophenyl)amino]-propyl ether (25-ANE), a hydrolytically stable photoaffinity analogue of 25-OH-D_3, and its radiolabeled counterpart, i.e., 25-hydroxyvitamin D_3 3β-3′-[*N*-(4-azido-2-nitro-[3,5-^{3}H]-phenyl)amino]propyl ether (^{3}H-25-ANE). We also describe the results of several studies with these analogues leading to the photoactivated covalent labeling of rDBP.

Experimental Procedures

All the synthetic operations were carried out in an argon atmosphere unless otherwise specified. Exposure to light was kept as low as possible when 25-ANE or ^{3}H-25-ANE was used. Purification of synthetic intermediates was performed on preparative (1000 μm) TLC plates purchased from Analtech Inc. (Newark, DE). 25-OH-D_3 was a gift from Upjohn Co. (Kalamazoo, MI). All the reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI), except lithium aluminum hydride and 4-fluoro-2-nitrophenyl azide, which were obtained from Alpha Ventron Corp. (Danvers, MA) and Pierce Chemical Co. (Rockford, IL), respectively. [26,27-^{3}H]-25-OH-D_3 (specific activity 20.6 Ci/mmol) and 4-fluoro-3-nitro-[2,6-^{3}H]phenyl azide (specific activity 50 Ci/mmol) were purchased from Amersham Corp., Arlington Heights, IL. HPLC solvents were from Burdick and Jackson (Muskegon, MI) and were used without purification. The autoradiography enhancer, EN^{3}HANCE was obtained from New England Nuclear (Boston, MA). HPLC analyses were carried out with a Waters HPLC system (Millipore Corp., Bedford, MA) consisting of a M40 pump, a U6K injector, and a UV/*vis* 440 detector. Detection of radioactivity of the HPLC effluent were carried out on a Radiomatic A-100 Flo-One Beta on-line radioactivity detector (Radiomatic Instruments and Chemical Co., Inc., Tampa, FL). UV spectra were obtained on a Perkin-Elmer UV/vis 552A spectrophotometer. Methanol was used as solvent unless otherwise specified. IR spectra were obtained on chloroform solutions of the compounds by using a Perkin-Elmer infrared spectrophotometer. High-field NMR spectra of the synthetic compounds were obtained on a Bruker 270-MHz instrument. Tetramethylsilane was used as the internal standard. Irradiation of the samples were carried out on ice in a shallow petri dish with a low-pressure mercury vapor lamp (Hanovia, obtained from Ace Glass Co., Vineland, NJ) from a height of 1–2 cm.

Rat DBP was obtained from pooled rat plasma and purified to homogeneity by a previously published method (Van Baelen & Bouillon, 1986). Rat serum was obtained from weanling male Holtzman rats (Holtzman Co., Madion, WI), maintained on a vitamin D deficient diet. The competitive binding assays (Chen et al., 1990) were carried out in a sodium barbital–sodium acetate buffer containing 0.1% affinity-purified BSA (Pierce Chemical Co., Rockford, IL), pH 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. All other studies were carried out in phosphate-buffered saline solution (PBS), pH 7.4.

Synthesis of 25-Hydroxyvitamin D_3 3β-3′-[N-(4-Azido-2-nitrophenyl)amino]propyl Ether (25-ANE) and 25-Hydroxyvitamin D_3 3β-3′-[N-(4-Azido-2-nitro-[3,5-^{3}H]-phenyl)amino]propyl Ether (^{3}H-25-ANE). (a) A solution of 25-OH-D_3 (40 mg) (A, Figure 1), acrylonitrile (66 μL), and Triton B (10 μL) in 2 mL of *t*-BuOH–CH_3CN (10:1) was stirred at 4 °C for 18 h, followed by addition of 10% HCl and extraction of the aqueous solution with ether. The product (B) was purified by preparative TLC (62% yield). NMR δ 0.58 (s, 3 H, C_{18}-CH_3), 0.95 (narrow d, 3 H, C_{22}-CH_3), 1.2 (s, 6 H, $C_{26,27}$-CH_3), 2.58 (t, 2 H, J = 6.3 Hz, CH_2CN), 3.6 (m, 1 H, C_3-H), 3.71 (m, 2 H, OCH_2); 4.81 and 5.03 (br s, 2 H, C_{19}-CH_2), and 6.12 (AB_q, 2 H, J = 11 Hz, $C_{6,7}$-CH). IR: weak absorption at 2270 cm^{-1}, characteristic of the nitrile group. The UV spectrum of this compound had characteristic

vitamin D absorptions with a maximum at 265 nm and a minimum at 228 nm.

(b) To an ice-cold slurry of $LiAlH_4$–$AlCl_3$ (15 equiv) in anhydrous ether was added a solution of (B) in ether under argon with stirring. The mixture was allowed to stir at room temperature for 1 h, followed by the addition of 1:1 water–ether and ice-cold 5 N KOH. The resulting mixture was extracted with ethyl acetate. The formation of amine (C) was quantitative by TLC. It was not purified any further and was used in the next step.

(c) A solution of the amine (C) (10 mg), 4-fluoro-3-nitrophenyl azide (12.4 mg), and triethylamine (10 μL) in 0.5 mL of DMSO was heated in the dark at 60 °C for 20 h, followed by the removal of the solvent under argon and purification of the product by preparative TLC. The yield of the orange product (D) was 73%. It was characterized by its UV spectrum in ethanol (strong absorption at 260 nm and a weak one at 450 nm) and by NMR: δ 0.54 (s, 3 H, C_{18}-CH_3), 0.94 (narrow d, 3 H, J = 5.91 Hz, C_{22}-CH_3), 1.21 (s, 6 H, $C_{26,27}$-CH_3), 3.5 (br m, 5 H, NH, N-CH_2, O-CH_2), 3.64 (m, 1 H, C_3-H), 4.81 and 5.03 (br s, 2 H, C_{19}-CH_2), 6.12 (AB_q, 2 H, J = 11 Hz, $C_{6,7}$), and aromatic Hs (3H) δ 6.9 (dd), 7.11 (dd), and 8.2 (m).

On the other hand, ^{3}H-25-ANE was synthesized by heating a solution of the amine (C) (100 μg), 4-fluoro-3-nitro-[2,6-^{3}H]phenyl azide (specific activity 50 Ci/mmol, 200 μCi), and triethylamine (5 μL) in 0.1 mL of ethanol at 60 °C in the dark for 4 days in a sealed vial, followed by the removal of the volatile material under argon and purification of the product by HPLC (Waters Radial Pak C_{18} column, 5% water in methanol). The fraction corresponding to a standard sample of 25-ANE was collected. The yield of ^{3}H-25-ANE was 40 μCi (25%).

Photolytic Degradation Studies of ^{3}H-25-ANE. Two samples, each containing 25-ANE (0.5 μg) and ^{3}H-25-ANE (33 000 cpm) in 20 μL of ethanol, were added to 200 μL of PBS (pH 7.4), and the solutions were incubated in the dark on ice for 15 min. Then, one sample (+UV) was irradiated for 2 min on ice with magnetic stirring, while the other was kept in the dark (–UV). Both the samples were then extracted with ethyl acetate followed by HPLC analysis (Econosil 5-μm column, Rainin Instruments, Woburn, MA, 2% 2-propanol in hexane, 1.5 mL/min.). Radioactivity associated with the chromatogram was monitored with an on-line radioactivity detector.

Competitive Binding Assays of 25-OH-D_3 and 25-ANE with Rat Serum DBP. Various amounts of either 25-OH-D_3 (0.125–8.0 nmol) or 25-ANE (0.40–161.33 nmol) dissolved in 20 μL of ethanol were added in triplicate to 0.5 mL of rat serum diluted 1:10 000 with the assay buffer, pH 8.6. ^{3}H-25-OH-D_3 (2000 cpm, specific activity 20.6 Ci/mmol), dissolved in 10 μL of ethanol, was added to each tube, and the solutions were incubated at 4 °C in the dark for 20 h, followed by the addition of 100 μL of ice-cold charcoal suspension to each tube and incubation on ice for 15 min. The tubes were then centrifuged (3000 rpm, 4 °C, 10 min). The supernatant from each tube was mixed with 10 mL of Instagel (Packard Instruments Co., Chicago, IL) and counted for radioactivity.

Determination of the Extent of Covalent Labeling of rDBP by ^{3}H-25-ANE as a Function of the Duration of UV Exposure. A solution of ^{3}H-25-ANE (50 Ci/mmol, 200 000 cpm, in 10 μL of ethanol) and purified rDBP (100 μg in 500 μL of PBS, pH 7.4) was incubated in the dark at 4 °C for 20 h. Aliquots (40 μL) of the incubated sample were irradiated (from a height of 2 cm) on ice for various time periods. Each time point was carried out in duplicate. After irradiation, each sample was diluted with 200 μL of PBS, and ice-cold ethanol (2 ml) was added to each sample. The samples were kept at 4 °C for 10 h, followed by centrifugation (3000 rpm, 15 min). The precipitated protein pellets were dissolved SDS in 0.1% N NaOH solution (100 μL) and heated on boiling water for 10 min. After cooling, the samples were mixed with scintillation cocktail (10 mL) and counted for radioactivity.

Photoaffinity Labeling Studies of Purified rDBP with ^{3}H-25-ANE. Three tubes, each containing 50 μL (50 μg) of purified rDBP solution in PBS (pH 7.4), were incubated at 4 °C in the dark with ethanolic (5 μL) solutions of (a) and (b) ^{3}H-25-ANE (200 000 cpm) alone (samples a and b) or ^{3}H-25-ANE (200 000 cpm) and 25-OH-D_3 (1 μg) (sample c). After 20 h of incubation, samples b and c were irradiated on ice with stirring for 2 min, while sample a was kept in the dark. Then each sample was dissolved in 25 μL of electrophoresis buffer (0.08 M Tris, 2 mM EDTA, 0.1 M DDT, 10% glycerol, and 0.2% bromophenol blue) and heated in boiling water for 2 min. The samples were then electrophoresed on an SDS–12% polyacrylamide gel (Laemli, 1970). Standard molecular weight markers (Bethesda Laboratories) were run alongside the actual samples. When the dye front reached the bottom of the gel, electrophoresis was stopped. The gel was fixed, treated with EN^{3}HANCE, dried, and finally exposed to Kodak X-OMat AR film at –78 °C for 5 days.

Results

The synthetic scheme, shown in Figure 1, produced high yield of 25-ANE, which was characterized by physicochemical means. The synthesis of ^{3}H-25-ANE was carried out in a similar fashion. The yield of ^{3}H-25-ANE was quite respectable despite the fact that the synthesis was carried out in a "carrier-free" fashion. ^{3}H-25-ANE was found to be radiochemically pure as determined by cochromatography (HPLC, 2% 2-propanol in hexane, 1.5 m/L min) of a sample of ^{3}H-25-ANE mixed with a standard sample of 25-ANE. In the HPLC chromatogram (Figure 2, –UV), the single UV peak of 25-ANE comigrated with a single radioactive peak with a retention time of 8.5 min. On the other hand, the photosensitive nature of ^{3}H-25-ANE was demonstrated by exposing it to UV radiation for 2 min, which caused total disintegration of the analogue (inset, Figure 2, +UV).

The specific binding affinity of 25-ANE for rDBP in crude rat serum was determined by competitive radioligand binding assays. The results of these assays (Figure 3) demonstrated that ^{3}H-25-OH-D_3, the natural substrate, was displaceable by the synthetic analogue (25-ANE) in a dose-dependent fashion. Furthermore, on a molar basis, the concentration of 25-ANE required to displace 50% of the ^{3}H-25-OH-D_3 bound to rDBP was 7.3 times greater than that required for 25-OH-D_3.

Evidence for the UV-activated cross-linking of the protein (rDBP) with ^{3}H-25-ANE was obtained by exposing a sample of rDBP, preincubated in the dark with ^{3}H-25-ANE, to UV for various time periods. The results (Figure 4) showed that the percentage of covalently attached radioactivity reached a maximum (14%) after 2 min of irradiation and then leveled off.

SDS–PAGE and autoradiographic analysis of a sample of rDBP, preincubated in the dark with ^{3}H-25-ANE, followed by UV exposure produced a radiolabeled band (M_r 51 000) as shown in lane 2, Figure 5. However, the extent of covalent labeling (of the protein) was insignificantly small when (i) the sample was not exposed to UV light (lane 1, Figure 5) or (ii) the irradiation was carried out in the presence of a large excess of 25-OH-D_3 (lane 3, Figure 5).

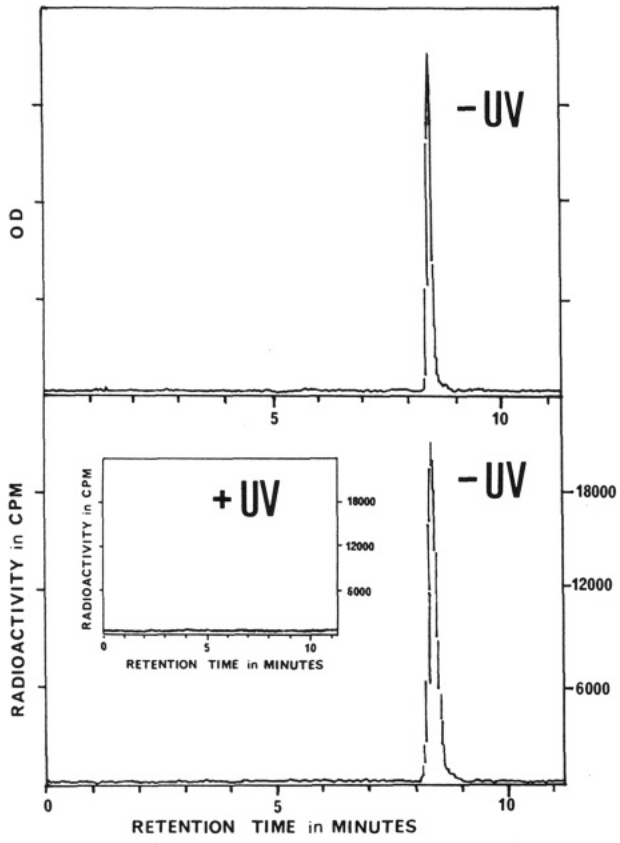

FIGURE 2: Two samples, each containing 25-ANE and ^{3}H-25-ANE, were incubated in the dark in PBS. One sample was exposed to UV for 2 min, while the other was kept in the dark. Both the samples were extracted with ethyl acetate and analyzed by HPLC (2% 2-propanol in hexane, 1.5 mL/min). Radioactivity was monitored by an on-line radioactivity detector. −UV, sample was not exposed to UV; +UV, sample was exposed to UV (inset).

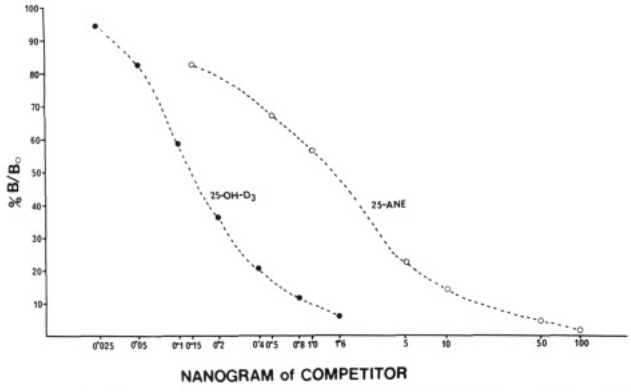

FIGURE 3: Competitive binding assays for 25-OH-D_3 and 25-ANE with rat serum DBP. Various concentrations of 25-OH-D_3 (●) and 25-ANE (○) were incubated in the dark with ^{3}H-25-OH-D_3 and rat serum in the assay buffer. Following the incubation, unbound sterols were removed by treatment with charcoal, and supernatants were counted for radioactivity.

Discussion

The goal of our study was to develop a synthetic photoaffinity analogue of 25-OH-D_3, capable of covalently labeling the 25-OH-D_3-binding site in rDBP when activated by UV light. Availability of such an analogue would potentially allow us to define this binding domain by tracking the covalently attached label in the peptide fragment(s) obtained by chemical and enzymatic cleavages of the protein labeled by this photoaffinity analogue.

There are seven disulfide bonds in rDBP (Cooke, 1986), which are responsible for its globular structure. We reasoned

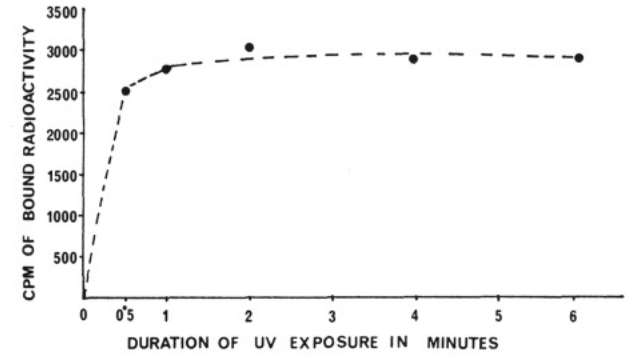

FIGURE 4: Determination of the UV-activated labeling of rDBP by ^{3}H-25-ANE as a function of time. A sample of rDBP was incubated in the dark with ^{3}H-25-ANE. Then aliquots were withdrawn and irradiated for various time periods. Protein from each sample (time point) was precipitated, redissolved, and counted for radioactivity. Counts for nonspecific binding (no UV exposure) was subtracted from the counts obtained from other samples.

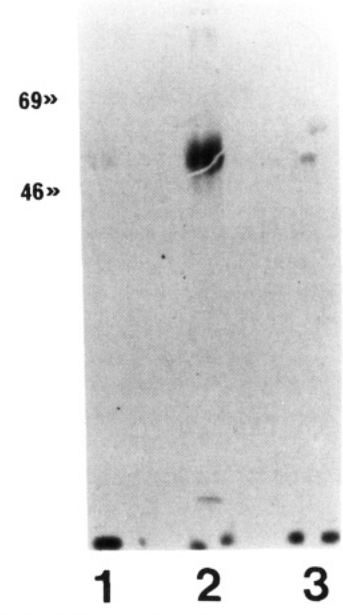

FIGURE 5: SDS–PAGE and autoradiographic analysis of samples of rDBP photoaffinity-labeled with ^{3}H-25-ANE. Two samples (a and b) of rDBP were incubated with ^{3}H-25-ANE in the dark; the third sample (c) contained a large excess of 25-OH-D_3 in addition to ^{3}H-25-ANE. Sample a was kept in the dark, while samples b and c were exposed to UV. All the samples were analyzed on an SDS–12% polyacrylamide gel. Lane 1, sample a; lane 2, sample b; and lane 3, sample c. Positions of standard molecular weight marker proteins are shown in the left.

that these disulfide bonds must be reduced and carboxymethylated to prevent the reassociation of the peptide fragments obtained by the chemical and enzymatic cleavages of the labeled protein.

We purified rDBP to homogeneity from pooled rat plasma by an established method (Van Baelen & Bouillon, 1986) and incubated it with ^{3}H-25-ANG, a photoaffinity analogue of 25-OH-D_3 (Ray et al., 1986), at 4 °C in the dark, followed by a short exposure to UV light. However, when the labeled protein was subjected to a standard procedure of carboxymethylation (8 M urea and 2 mM DTT, pH 8.5, plus iodoacetamide, followed by dialysis against 50 mM NH_4CO_3) (Allen, 1981), there was an almost complete loss of the radiolabel. This was possibly due to the vulnerability of the ester bond in ^{3}H-25-ANG in a strongly basic pH (results not shown). Since the tracer atoms in ^{3}H-25-ANG are located in the side chain of the 25-OH-D_3 part of the molecule (Figure

1, inset), cleavage of the ester bond led to a significant loss of radioactivity. This observation prompted us to develop a second-generation photoaffinity analogue of 25-OH-D_3 that would be stable under a wide pH range.

We chose to anchor the photoactive group to the 25-OH-D_3 molecule via a relatively stable ether bond as in the case of 25-ANE. The synthesis of 25-ANE (Figure 1) employed base-catalyzed Michael addition of the 3β-hydroxyl group of 25-OH-D_3 across the double bond of acrylonitrile, the well-known Michael acceptor. The nitrile group, via its reduction, provided a chemically reactive primary amino group, which was coupled to 4-fluoro-3-nitrophenyl azide in a nucleophilic displacement reaction. The product, 25-ANE, consisted of a photoactivable group attached to the parent steroid molecule via a stable ether bond. The short length of the spacer arm made the analogue (25-ANE) suitable for our photoaffinity labeling studies.

The synthesis of ^{3}H-25-ANE followed a procedure similar to that used for 25-ANE. The specific activity of ^{3}H-25-ANE was high (50 Ci/mmol), thus making it suitable for autoradiographic detection in labeling experiments.

The desired chemical stability of ^{3}H-25-ANE was determined by subjecting an ethanolic solution of ^{3}H-25-ANE to the conditions of carboxymethylation as described above. HPLC analysis of the ethyl acetate extract of the reaction mixture showed that ^{3}H-25-ANE was fully stable under these conditions (results not shown).

We carried out studies to demonstrate the photosensitive nature of ^{3}H-25-ANE (Figure 2), as well as to show that this analogue is capable of covalently labeling rDBP when activated by UV light (Figure 4; Figure 5, lanes 1 and 2). On the other hand, we have shown that 25-ANE could compete with ^{3}H-25-OH-D_3 for the binding of the latter to rDBP (Figure 3). These results, in combination, indicate that 25-ANE, a synthetic analogue of 25-OH-D_3, is capable of entering the 25-OH-D_3 binding pocket in rDBP and covalently labeling it when activated by UV. More conclusive evidence was obtained by carrying out the photoaffinity labeling experiment in the presence of a large excess of 25-OH-D_3. We reasoned that since 25-OH-D_3 binds to rDBP more efficiently than 25-ANE, it would be possible to eliminate most or all of the noncovalently bound ^{3}H-25-ANE from the binding site (of rDBP) by incubating in the presence of a large excess of 25-OH-D_3. A small amount of labeling is, however, expected, even in the presence of a large excess of 25-OH-D_3. This is due to the adherence of some ^{3}H-25-ANE molecules, displaced by 25-OH-D_3 from the binding pocket, to the protein by hydrophobic interactions. The result of the electrophoresis–fluorography experiment (Figure 5, lanes 2 and 3) convincingly attests to this reasoning.

In conclusion, the studies described in this paper demonstrate that 25-ANE, a hydrolytically stable second-generation photoaffinity analogue of 25-OH-D_3, is capable of covalently labeling the 25-OH-D_3 binding site in rDBP upon UV exposure. These results could be coupled with the knowledge of the full-length primary structure of rDBP (Cooke, 1986), to identify the 25-OH-D_3 binding region in rDBP, as well as the points of contact within this region. Work is currently under way in our laboratories to attain this goal.

Registry No. 25-ANE, 133191-08-9; ^{3}H-25-ANE, 133191-09-0; 25-OH-D_3, 19356-17-3; 25-OH-D_3 NC-CH_2-CH_2 ether derivative, 133191-10-3; 4-fluoro-2-nitrophenyl azide, 67312-96-3.

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