Effects of Protein Modification Procedures on the Interaction between 25-Hydroxyvitamin D and the Human Plasma Binding Protein for Vitamin D and Its Metabolites†

Masanobu Kawakami and DeWitt S. Goodman*

ABSTRACT: Studies were conducted to explore the effects of protein modification procedures on the interaction between 25-hydroxyvitamin D_3 [25(OH)D_3] and the human plasma transport protein for vitamin D and its metabolites (DBP). DBP is identical with the human plasma group-specific component (Gc) protein. The effects of progressive modification of lysine and of arginine residues, by reductive methylation and with cyclohexanedione, respectively, of iodination of tyrosine residues, of reduction and alkylation of disulfide bonds, and of exposure to 6 M guanidine hydrochloride were investigated. Effects on binding properties were compared with those on DBP immunoreactivity. Progressive modifications of lysine, arginine, and tyrosine residues all resulted in progressive decreases in the binding activity of DBP for 25(OH)D_3 without affecting immunoreactivity. The patterns of loss of binding activity differed among these three kinds of modifications. Thus, modification of 6 (of a total of 14) arginine residues did not affect binding activity, whereas further arginine modifications resulted in a progressive decrease in binding. Iodination of DBP beyond 1 atom of iodine per molecule of protein led to a fairly marked decline in binding activity, whereas a more gradual decrease in binding was seen with progressive lysine modification. In the presence of 6 M guanidine hydrochloride, DBP displayed virtually no binding activity for 25(OH)D_3. The effects of guanidine on the association of DBP with its ligand were, however, completely reversible. Reductive alkylation of disulfide bonds profoundly decreased both the 25(OH)D_3 binding activity and the immunoreactivity of DBP. The results suggest that a limited number of disulfide bonds play a major and critical role in maintaining the stable, three-dimensional structure of DBP that is required for its 25(OH)D_3 binding activity and for its immunoreactivity.

Vitamin D and its oxygenated metabolites circulate in plasma bound to a specific transport protein, the binding protein for vitamin D and its metabolites (DBP).[1] It is now well recognized that human DBP is identical with the human plasma group-specific component (Gc) protein (Daiger et al., 1975; Haddad & Walgate, 1976a; Bouillon et al., 1976; Imawari & Goodman, 1977). DBP/Gc protein has a molecular weight of ~52 000 (Imawari et al., 1976) and contains one binding site for one molecule of vitamin D related sterol per molecule of DBP. The normal concentration of DBP in plasma is on the order of 300–600 μg/mL (Imawari & Goodman, 1977; Haddad & Walgate, 1976b; Bouillon et al., 1977).

Gc proteins display genetic polymorphism and have been investigated extensively as a marker for genetic studies during the past 15–20 years (Cleve, 1973; Giblett, 1969). Three common phenotypes (Gc 1-1, Gc 2-1, and Gc 2-2) have been found in somewhat varying proportions throughout the world; a moderately large number of uncommon and rare Gc genetic variants and phenotypes have also been observed. The major components of DBP/Gc protein appear to occur as a result of the effects of two autosomal alleles at a single locus (Putman, 1977; Svasti et al., 1979). Information about the molecular basis for the three major forms of human DBP/Gc proteins was provided recently by the work of Svasti et al. (1979).

We have recently reported the results of quantitative studies of the interaction of several vitamin D related sterols with different genetic variants of DBP/Gc protein (Kawakami et al., 1979). The ligands tested included vitamin D_3, 25(OH)D_3, 1,25$(OH)_2D_3$, and 24(*R*),25$(OH)_2D_3$. The common genetic variants of DBP/Gc protein, and several uncommon genetic variants also studied, all appeared to have similar binding properties for the vitamin D related sterols (Kawakami et al., 1979).

The studies reported here were undertaken in order to obtain more information about some of the chemical and physical properties of DBP and about structural factors affecting its interaction with 25(OH)D_3. To this end, the effects of several protein modification procedures on the interaction of DBP with 25(OH)D_3 were explored. The procedures investigated included progressive modification of lysine and of arginine residues with specific reagents for these residues, iodination of tyrosine residues, and reduction and alkylation of disulfide bonds. The effects of protein modification on the binding properties of DBP were compared with effects on DBP immunoreactivity.

Materials and Methods

Isolation of DBP. DBP was isolated from pooled human plasma by methods similar to those described previously from this laboratory (Imawari et al., 1976). Immunoreactivity against monospecific anti-human DBP antiserum (Imawari & Goodman, 1977) was used as a marker for DBP during the course of its isolation. The sequence of procedures used to isolate DBP for the studies reported here included column chromatography on SP-Sephadex, then on DEAE-cellulose, and then on immobilized Cibacron Blue F3GA (Pierce), followed by gel filtration on Sephacryl S-200. During chromatography on SP-Sephadex, holo-DBP [i.e., DBP containing

† From the Department of Medicine, Columbia University College of Physicians and Surgeons, New York, New York 10032. *Received March 18, 1981.* This research was supported by Grant AM-05968 from the National Institutes of Health.

[1] Abbreviations used: DBP, binding protein for vitamin D and its metabolites; Gc protein, group-specific component protein; 25(OH)D, 25-hydroxyvitamin D; 1,25$(OH)_2$D, 1,25-dihydroxyvitamin D; 24,25-$(OH)_2$D, 24,25-dihydroxyvitamin D; DTE, dithioerythritol; DTT, dithiothreitol; PBS, phosphate-buffered saline; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

0006-2960/81/0420-5881$01.25/0

a molecule of bound 25(OH)D_3, identified by the addition of a tracer amount of [^{3}H]25(OH)D_3 to the plasma before chromatography (Imawari et al., 1976)] was eluted first, followed by the main mass of DBP present as apo-DBP in plasma. The fractions comprising the peak of eluted apo-DBP were pooled and used for further fractionation. During chromatography of the partially purified apo-DBP on immobilized Cibacron Blue, partial separation of the DBP/Gc isoforms occurred, characterized by the elution of two partly resolved peaks of DBP immunoreactivity. The first peak of DBP consisted predominantly of the Gc 1 isoform, whereas the second peak consisted of the Gc 2 isoform. The final yield of isolated apo-DBP was ~15 mg from each liter of the initial pooled plasma. The relative yields of the two isoforms (Gc 1/Gc 2) were approximately 2:1. The studies reported here were conducted with a preparation of purified apo-DBP consisting largely of the Gc 1 isoform. The purity and Gc type of the final purified protein preparations were established, respectively, by polyacrylamide disc gel electrophoresis (Imawari et al., 1976; Daiger & Cavalli-Sforza, 1977) and by immunoelectrophoresis (Hirshfeld, 1962).

Iodination of DBP. DBP was labeled with carrier-free Na^{125}I (New England Nuclear) in 1 M glycine buffer at pH 9.0 and the ICl method of McFarlane (1958). Four equal-sized portions of a solution of DBP (1.2 mg/mL) were reacted with progressively increasing amounts of ICl, with molar ratios (of ICl/DBP) of 1.4, 4.3, 11.4, and 22.9, respectively, in the four reaction solutions. The final pH of each reaction mixture was 8.5–9.0. Free iodine was removed by extensive dialysis against phosphate-buffered saline (PBS, 0.01 M potassium phosphate buffer, pH 7.4, and 0.145 M NaCl) at 4 °C. Analysis of the iodinated DBP preparations by gel filtration on a small column of Sephadex G-25 and by polyacrylamide disc gel electrophoresis showed that more than 97% of the radioactivity was bound to the protein after dialysis. The number of microgram atoms of iodine incorporated per micromole of protein was calculated for each preparation from the specific radioactivity of the iodine in the reaction mixture and from the measured incorporation of radioactivity per micromole of protein.

Reductive Methylation of DBP. Reductive methylation of the ϵ-amino groups of lysine residues was performed at 0 °C with sodium borohydride and [^{14}C]formaldehyde (New England Nuclear), as described by Means & Fenny (1968). Increasing levels of modification of the protein were achieved by repeated addition (total of two to six repeated additions), at 1-min intervals, of solutions of borohydride and formaldehyde to the reaction mixtures. The molar amounts of sodium borohydride added were about half those of formaldehyde. Five reaction mixtures were prepared with molar ratios of formaldehyde to lysine residues (of DBP) of 0.8, 1.6, 4.1, 8.3, and 21.6, respectively. After the final addition of sodium borohydride, each reaction mixture was dialyzed against PBS at 4 °C to remove unreacted reagents. The number of methyl groups incorporated per molecule of DBP was calculated for each preparation from the specific radioactivity of the formaldehyde in the reaction mixture and from the measured incorporation of radioactivity per micromole of protein.

Cyclohexanedione Modification of DBP. Arginine residues of DBP were selectively modified with 1,2-cyclohexanedione according to the method of Patthy & Smith (1975). Five samples of DBP, in solution in 0.2 M sodium borate buffer pH 9.0, were incubated with progressively increasing amounts of 1,2-cyclohexanedione at 37 °C for 2 h in sealed tubes under N_2. The molar amounts of 1,2-cyclohexanedione varied from 5 to 80 times the molar amounts of arginine residues (of the DBP) (with molar ratios of cyclohexanedione to arginine residues of 5, 10, 20, 40, and 80, respectively). After completion of the reaction, half of each reaction mixture was dialyzed against 10 mM sodium borate buffer, pH 7.7, and used for studies of 25(OH)D_3 binding and DBP immunoreactivity. The other half of the reaction mixture was used for amino acid analysis; the number of arginine residues modified was calculated from the number of arginine residues remaining intact. Intact arginine residues were regenerated from the modified arginine residues with hydroxylamine (Patthy & Smith, 1975).

Sulfhydryl and Disulfide Assays. The content of free sulfhydryl groups in DBP was estimated by the method of Ellman (1959). The reaction was carried out under N_2, with solutions that had been thoroughly gassed with N_2 before use. With cysteine as a standard, the observed molar extinction coefficient for the nitrothiophenolate product of the reaction at 412 nm was $(1.18 \pm 0.03) \times 10^4$ (mean ± SD). This value is very close to that of 1.19×10^4 reported by Robyt et al. (1971).

The disulfide content of DBP was assayed by the method of Zahler & Cleland (1968). DBP (0.52 mg/mL) was reduced with 2 mM dithioerythritol (DTE) at pH 9.0 in the presence or absence of 6 M guanidine hydrochloride. The resulting –SH groups were measured after a 4- or 20-h reduction at room temperature. The procedure was carried out under N_2 by employing N_2-saturated solutions as described above. With cystine as a standard, the observed molar extinction coefficient for the nitrothiophenolate product of the reaction was 1.23×10^4. This value was used to calculate the number of monothiols produced by reduction.

Reductive Alkylation of Disulfide Bonds of DBP. Progressively increasing numbers of disulfide bonds were reduced with increasing concentrations of dithiothreitol (DTT), and resulting sulfhydryl groups were alkylated with monoiodo[1-^{14}C]acetic acid (Cleland, 1964; Hirs, 1967). Three reaction mixtures were prepared, each containing 100 μL of DBP (~ 8.5 mg/mL) in 1 M Tris-HCl buffer, pH 8.0, containing 6 M guanidine hydrochloride, plus solid DTT added in molar ratios (DTT/DBP) of 1.0, 2.8, and 6.9. Reduction was allowed to proceed at room temperature for 4.5 h under N_2. Alkylation of the reduced protein was then carried out with a 55-fold molar excess (compared to the molar amount of DBP) of monoiodo[1-^{14}C]acetic acid (New England Nuclear; recrystallized with nonradioactive monoiodoacetic acid to yield final specific activity of 0.30 mCi/mmol) (Hirs, 1967). The alkylated protein was separated from reaction reagents by gel filtration on a column of Sephadex G-25 equilibrated with 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and was then dialyzed against the same buffer. The number of alkylated sulfhydryl groups [i.e., of (carboxymethyl)cysteine residues present] was calculated for each preparation from the specific radioactivity of the monoiodoacetic acid in the reaction mixture and from the measured incorporation of radioactivity per micromole of protein.

Effect of Guanidine Hydrochloride. The 25(OH)D_3 binding properties of DBP were examined after exposure of the protein to 6 M guanidine hydrochloride. DBP was dissolved in 0.04 M Tris-HCl buffer, pH 8.1, containing 6 M guanidine hydrochloride (0.4 mg of DBP/mL) and was allowed to stand at 5 °C for 3 days. A 10-fold molar excess of [^{3}H]25(OH)D_3 was added (in 25 μL of ethanol) to 0.5-mL samples of this DBP solution, and the solution then incubated

for 5 h at room temperature. Separate control experiments indicated that this concentration (5%) of ethanol does not affect binding of ligand to protein. Protein-bound [^{3}H]25-(OH)D_3 was separated from free ligand (unbound to the protein) by gel filtration on Sephadex G-50. Gel filtration was carried out both with buffer containing 6 M guanidine hydrochloride and with buffer devoid of guanidine hydrochloride.

In another experiment, DBP was dissolved in buffer containing 6 M guanidine hydrochloride. After 3 days at 5 °C the guanidine hydrochloride was removed by gel filtration on Sephadex G-50 (fine), with 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA as eluting buffer, or by extensive dialysis against the same buffer. After dialysis or gel filtration the DBP was assayed for its binding of 25(OH)D_3 (Kawakami et al., 1979).

DBP Binding Properties and Immunoreactivity. The binding properties of the various modified DBP preparations for 25(OH)D_3 were examined by using the DEAE-filter assay method described previously (Kawakami et al., 1979). In order to quantitatively estimate the relative binding activity of a given preparation, we equilibrated ~0.5 μg (1×10^{-11} mol) of modified or unmodified DBP in 0.5 mL of buffer with an equimolar amount of [^{3}H]25(OH)D_3 added in 10 μL of ethanol. The assay buffer consisted of 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and human plasma lipoproteins of density < 1.063 g/mL, as described previously (Kawakami et al., 1979). All assays were conducted in duplicate with close agreement in results obtained between duplicate pairs. The results presented here represent the mean of these duplicate values. The amount of [^{3}H]25(OH)D_3 bound to the preparation of modified DBP was compared to that bound to unmodified DBP when assayed together under identical conditions. The result, for each preparation, was expressed in percent terms, to provide a measure of the relative binding activity of the modified DBP preparation.

The immunoreactivity of the modified DBP preparations was quantitatively estimated by the radial immunodiffusion assay as described previously (Imawari & Goodman, 1977). The result, for each preparation, was expressed as the percent of the immunoreactivity of the intact (unmodified) DBP, for samples of identical protein concentration.

Spectral Studies. Absorbances and absorption spectra were measured with a Gilford Model 2400 or a Beckman DB spectrophotometer. A study was conducted to compare the absorption spectra of apo- and of holo-DBP [i.e., to compare apo-DBP with the DBP–25(OH)D_3 complex]. For this study, holo-DBP was prepared by incubating apo-DBP (1 mg/mL in PBS) with a 3.5-fold molar excess of [^{3}H]25(OH)D_3 (added in ethanol solution; final concentration of ethanol 5% v/v). The 25(OH)D_3–DBP complex was separated from free ligand (unbound to the protein) by gel filtration on Sephadex G-100. The amount of 25(OH)D_3 bound to the protein was calculated from the specific radioactivity of [^{3}H]25(OH)D_3 and the amount of radioactivity bound per micromole of DBP.

A sample of purified apo-DBP was desalted by extensive dialysis against distilled water, then lyophilized, and dried over P_2O_5 under vacuum for 4.5 days. A precisely weighed portion of this DBP was dissolved in PBS containing 1 mM EDTA. The extinction coefficient of the apo-DBP at 280 nm ($E^{1\%}_{1cm}$) was estimated from the absorbance of this solution, and its protein concentration was determined gravimetrically. The protein concentration was also measured by the method of Lowry et al. (1951), with purified human serum albumin as a standard. The extinction coefficient ($E^{1\%}_{1cm}$) of the 25-(OH)D_3–DBP complex was estimated from the absorbance

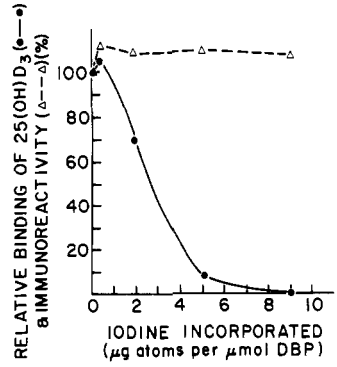

FIGURE 1: Effect of iodination on the interaction of DBP with 25-hydroxyvitamin D_3 and on DBP immunoreactivity.

of the holo-DBP solution (see above) at 280 nm and the protein concentration of that solution determined by the method of Lowry et al. (1951).

Other Methods. The amino acid compositions of chemically modified DBP preparations were determined after hydrolysis of 50–100 μg of protein in 6 N HCl at 110 °C for 24 h. Analyses were performed (Spackman et al., 1958) by Dr. Stephen Birken of this medical school, with a Beckman 121 MB amino acid analyzer. For protein samples with modified arginine residues, 20 μL of mercaptoacetic acid was added before protein hydrolysis to prevent regeneration of arginine during acid hydrolysis (Patthy & Smith, 1975).

For radioassay for ^{3}H or for ^{14}C, samples were dissolved in ScintiVerse and assayed in a Packard Model 3003 liquid scintillation spectrometer. ^{125}I radioactivity was assayed with a Packard Model 5219 counter. The dual-channel simultaneous equation method was employed for assay of ^{3}H in the presence of ^{125}I or ^{14}C.

Results

Iodination of DBP. Four portions of DBP were progressively iodinated with increasing amounts of ICl (see Materials and Methods). The numbers of microgram atoms of iodine incorporated per micromole of DBP in the four preparations were respectively 0.35, 1.9, 5.1, and 9.1.

Mixtures of iodinated DBP and of untreated DBP were analyzed by polyacrylamide disc gel electrophoresis, and the mobility of the iodinated DBP preparations was determined by the distribution of radioactivity on the sliced gel. The DBP preparations containing ≤5.1 μg-atoms of iodine/μmol of DBP showed mobility that was identical with that of untreated DBP. The DBP preparation containing 9.1 μg-atoms of iodine/μmol of DBP showed the same mobility as untreated DBP, but the band of radioactivity was wider and distributed more broadly than the band of stained protein.

Figure 1 shows the results of the studies on the relative binding of 25(OH)D_3 and on the relative immunoreactivity of the iodinated DBP preparations. Immunoreactivity was unchanged by iodination, in all of the four iodinated preparations studied. In contrast, iodination of DBP beyond a level of ~1 μg-atom of iodine/μmol of DBP substantially reduced the relative binding activity of DBP for 25(OH)D_3. Thus, while the binding of 25(OH)D_3 was essentially unchanged (from that of untreated DBP) in the preparation containing 0.35 μg-atom of iodine/μmol of DBP, iodination at a level of 1.9 μg-atoms of iodine/μmol of DBP resulted in an ~30% decrease in binding of 25(OH)D_3. The binding of 25(OH)D_3 was decreased more than 90% by iodination at a level of 5.1 μg-atoms of iodine/μmol of DBP and was virtually abolished by iodination at the highest level studied.

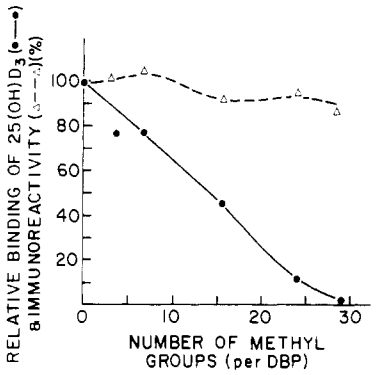

FIGURE 2: Effect of reductive methylation of lysine residues on the interaction of DBP with 25-hydroxyvitamin D_3 and on DBP immunoreactivity.

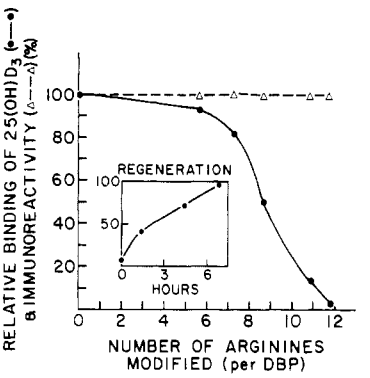

FIGURE 3: Effect of modification of arginine residues with 1,2-cyclohexanedione on the interaction of DBP with 25-hydroxyvitamin D_3 and on DBP immunoreactivity. The insert shows the relative binding activity of modified DBP after regeneration of arginine residues with hydroxylamine.

Reductive Methylation of DBP. Five portions of DBP were reacted with increasing amounts of borohydride and formaldehyde in order to reductively methylate progressively increasing numbers of lysine residues per molecule of protein. The number of methyl groups incorporated per molecule of DBP increased linearly with increasing amounts of formaldehyde added, up to the addition of 8.3 times the molar concentration of lysine residues. At this level of added formaldehyde, 24 methyl groups were incorporated per molecule of DBP. On addition of still further formaldehyde, the efficiency of methylation was decreased, so that only 29 methyl groups were incorporated per molecule of DBP when the molar ratio of formaldehyde to lysine residues was increased to 21.6.

Figure 2 shows the relative binding of $25(OH)D_3$ and the relative immunoreactivity of the reductively methylated DBP preparations. Immunoreactivity was only minimally affected by reductive methylation, in all five preparations studied. With progressively increasing lysine modification, however, a progressive decline in relative binding activity for $25(OH)D_3$ was observed. The decline in relative binding of $25(OH)D_3$ was roughly proportional to the number of methyl groups incorporated, throughout the range of values studied. Thus, the decrease in $25(OH)D_3$ binding was ~50% when about 14 to 15 methyl groups were incorporated per molecule of DBP. $25(OH)D_3$ binding was decreased more than 95% in the DBP preparation containing 29 methyl groups per molecule of protein.

As a control for this study, samples of DBP were reacted with sodium borohydride alone (in the absence of formaldehyde) over the range of concentrations used in this study. These treated DBP preparations showed a slight depression of $25(OH)D_3$ binding (90 ± 1% of untreated DBP, $n = 8$) that was not related to the concentration of borohydride employed.

Arginine Modification. Five portions of DBP were reacted with increasing amounts of cyclohexanedione in order to modify progressively increasing numbers of arginine residues per molecule of protein. Of the 14 estimated arginine residues present per molecule of DBP (Imawari et al., 1976), ~6 appeared to be readily susceptible to modification with cyclohexanedione, whereas the remainder appeared less accessible to the modification reaction. Thus, addition of a 5-fold molar excess of cyclohexanedione (compared to the concentration of arginine residues) resulted in an average of 5.7 arginine groups being modified per molecule of DBP. Addition of twice as much cyclohexanedione (a 10-fold molar excess) resulted in only a small further increase in the number of arginine groups modified (to 7.3), and addition of a 20-fold molar excess of cyclohexanedione resulted in only 8.8 arginine groups being modified.

The effects of arginine modification on $25(OH)D_3$ binding appeared to relate to the relative susceptibility of the arginine residues to the modification reaction. Thus, as shown in Figure 3, modification of 5.7 arginine residues per molecule of DBP had very little effect on the binding of $25(OH)D_3$ by DBP. Progressively further modification of the arginine residues of DBP resulted, however, in a progressive loss of $25(OH)D_3$ binding activity. Thus, $25(OH)D_3$ binding was decreased ~50% in the DBP preparation with 8.8 arginine residues modified, and modification of 11 arginine residues resulted in a 95% decrease in $25(OH)D_3$ binding. In contrast, immunoreactivity was fully maintained regardless of the degree of arginine modification (Figure 3).

The effects of arginine modification of $25(OH)D_3$ binding were fully reversible (Figure 3, insert). Thus, regeneration of arginine residues with hydroxylamine restored more than 95% of the $25(OH)D_3$ binding activity to the most extensively modified (11.8 arginine residues modified per molecule) preparation of DBP.

Sulfhydryl and Disulfide Analyses of DBP. Samples of DBP were assayed for their detectable –SH and S–S content, as described under Materials and Methods. Less than one free –SH group was detected in DBP, when the assay was carried out both in the absence of guanidine (0.78 ± 0.34, mean ± SD, $n = 3$) and in the presence of 6 M guanidine hydrochloride (0.42 ± 0.21, mean ± SD, $n = 3$). We conclude that DBP probably has one monothiol group per molecule but that it is not fully accessible to the assay reagents, even in the presence of 6 M guanidine.

In the absence of denaturing agents, DBP was quite resistant to reduction of disulfide bonds by DTE. Under these conditions no disulfide bonds were reduced after 4 h and only approximately one after 20 h of incubation with DTE. Assay for sulfhydryl content after reduction of disulfide bonds with DTE for 4 or 20 h in the presence of 6 M guanidine hydrochloride yielded an estimate of 15 –SH groups per molecule of protein.

Reductive Alkylation of Disulfide Bonds. Three portions of DBP were reacted with increasing concentrations of DTT and the resulting sulfhydryl groups alkylated with iodo[1-^{14}C]acetic acid. The numbers of micromoles of acetic acid incorporated per micromole of DBP [i.e., the number of micromoles of (carboxymethyl)cysteine residues formed] were found to be 1.1, 5.1, and 13, respectively, for the three preparations.

Table I shows the relative binding activity for $25(OH)D_3$ and the relative immunoreactivity of the reduced and alkylated DBP preparations. Reduction and alkylation of disulfide bonds

Table I: Effect of Reductive Alkylation of Disulfide Bonds on Interaction of DBP with 25-Hydroxyvitamin D_3 and on DBP Immunoreactivity

no. of CM[a] groups (per DBP)	rel binding of 25(OH)D_3 (%)	rel immunoreactivity (%)
0	(100)	(100)
1.1	12	35
5.1	<1	<1
13	0	0

[a] CM, carboxymethyl.

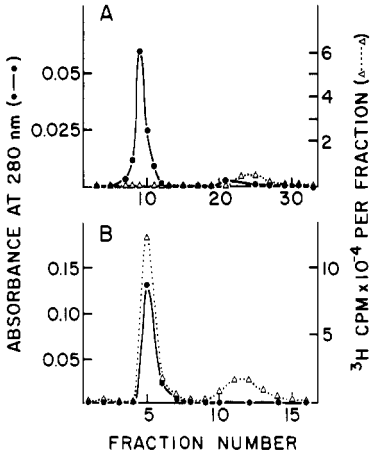

FIGURE 4: Effect of 6 M guanidine hydrochloride on the interaction of DBP with 25-hydroxyvitamin D_3. A solution of DBP containing 6 M guanidine hydrochloride, which had been incubated with [^{3}H]25(OH)D_3 (see Materials and Methods), was gel filtered on either (panel A) a column of Sephacryl S-200 (1 × 25.5 cm) using an eluting buffer of 50 mM Tris–glycine, pH 8.1, containing 6 M guanidine hydrochloride or (panel B) a column of Sephadex G-50 (1 × 15 cm, fine) equilibrated with and using a buffer devoid of guanidine hydrochloride. The eluted fractions (1 mL each) were assayed for absorbance at 280 nm (as a measure of protein, i.e., DBP) and for ^{3}H [as a measure of 25(OH)D_3].

resulted in a marked decrease of both 25(OH)D_3 binding and of immunoreactivity. Thus, the DBP preparation that had been reacted with an equimolar amount of DTT and which contained 1.1 carboxymethyl group per molecule of protein showed only 12% of the 25(OH)D_3 binding activity and only 35% of the immunoreactivity of the untreated protein. The DBP preparations which had been more extensively reduced and alkylated (5 or 13 carboxymethyl groups per molecule) showed virtually complete loss of both 25(OH)D_3 binding activity and of immunoreactivity.

Effect of Guanidine Hydrochloride. The effects of guanidine hydrochloride on the binding of 25(OH)D_3 by DBP were explored in several ways. In the first experiment, DBP was exposed to 6 M guanidine hydrochloride for several days. [^{3}H]25(OH)D_3 was then added, followed by gel filtration. Figure 4A shows the elution of protein (DBP) and of ^{3}H [representing 25(OH)D_3] when gel filtration was carried out with an eluting buffer containing 6 M guanidine hydrochloride. Under these conditions, almost no [^{3}H]25(OH)D_3 was eluted together with (i.e., bound to) DBP. In contrast, when gel filtration was carried out with an eluting buffer devoid of guanidine hydrochloride (Figure 4B), [^{3}H]25(OH)D_3 was eluted together with DBP, as a 1:1 molar DBP–25(OH)D_3 complex. As a control, when the same amount of [^{3}H]25(OH)D_3 was dispersed in buffer without DBP (and without guanidine hydrochloride) and gel filtered on the same column, almost no ^{3}H was detected in the fractions where DBP would have been eluted.

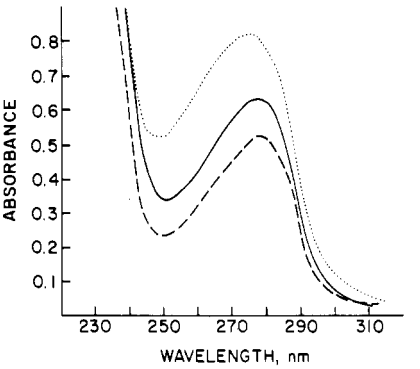

FIGURE 5: Absorption spectra of apo-DBP and of the DBP–25(OH)D_3 complex (holo-DBP). The bottom curve shows the spectrum of a solution of apo-DBP of concentration exactly 1 mg/mL; the middle curve shows the spectrum of a solution of the DBP–25(OH)D_3 complex of the same protein concentration. Absorption spectra were recorded on four solutions of apo-DBP of different concentrations. Each spectrum was normalized to a concentration of exactly 1 mg/mL by using the measured value for the $E^{1\%}_{1cm}$ at 280 nm (see text). The mean of the four normalized spectra is shown as the bottom curve in the figure. A similar procedure was employed to obtain the spectrum of the DBP–25(OH)D_3 complex (middle curve). The top curve shows the calculated spectrum obtained by adding together the spectra of apo-DBP and of 25(OH)D_3 in solution in ethanol (see text).

In another experiment, DBP was exposed to guanidine hydrochloride for 3 days, and the guanidine hydrochloride then removed by dialysis or by gel filtration. In both preparations, the binding affinity of DBP for 25(OH)D_3 was fully restored after removal of the guanidine hydrochloride.

Spectral Studies. Figure 5 shows the absorption spectra for apo-DBP (bottom curve) and for the DBP–25(OH)D_3 complex (holo-DBP) (middle curve), for DBP solutions at a concentration of precisely 1 mg/mL. The spectra dawn represent the mean values for spectra obtained in four different experiments.

The absorption spectrum of 25(OH)D_3 in ethanol was obtained. A "theoretical" spectrum was then calculated for the DBP–25(OH)D_3 complex, which would have been obtained if the absorption spectrum of 25(OH)D_3 bound to DBP were the same as that of 25(OH)D_3 alone in solution in ethanol. This calculated spectrum [i.e., the sum of the spectra for apo-DBP and that for 25(OH)D_3] is shown as the top curve in Figure 5. Throughout the entire absorbance range studied, the observed absorbance of the DBP–25(OH)D_3 complex was less than that calculated for the theoretical spectrum of the complex. The observed absorbance ratios (A_{280nm}/A_{260nm}) were 1.52 for apo-DBP and 1.47 for the DBP–25(OH)D_3 complex.

The measured $E^{1\%}_{1cm}$ at 280 nm for apo-DBP was 5.21 ± 0.10 (mean ± SD, n = 5) when the DBP protein concentration was measured gravimetrically and 4.56 ± 0.15 when the protein concentration was measured by the method of Lowry et al. (1951). The corresponding $E^{1\%}_{1cm}$ values at 280 nm for the DBP–25(OH)D_3 complex were 6.2 and 5.6. A theoretical value of 7.8 can be calculated for the DBP–25(OH)D_3 complex from the sum of the absorbance at 280 nm of apo-DBP in aqueous solution and of 25(OH)D_3 in ethanol (see Figure 5).

Discussion

The human DBP molecule consists of a single polypeptide chain with a molecular weight of ~52 000 (Imawari et al., 1976; Svasti et al., 1979) and with one binding site for one molecule of a vitamin D related sterol per molecule of protein. In the studies reported here, chemical modifications of apo-DBP were carried out in order to determine the accessibility of some of its functional groups to modifying agents and the

effects of such modifications on the interaction of DBP with 25(OH)D_3 and on DBP immunoreactivity.

DBP/Gc protein contains on the order of 40 lysine residues per molecule of protein (Imawari et al., 1976; Svasti et al., 1979). These residues were modified progressively by reductive methylation, a procedure which does not alter the positive charge on the ϵ-amino group of lysine. The modification reaction yields both mono- and dimethyllysine (Means & Fenney, 1968; De La Llosa et al., 1974), so that one cannot determine precisely the number of lysine residues modified from the number of methyl groups incorporated into the protein. With progressively increasing lysine modification, from 0 to 29 methyl groups incorporated per molecule of protein, a progressive decrease in DBP binding activity for 25(OH)D_3 was observed. The results suggest that lysine groups in general play a role in those aspects of DBP structure that are related to 25(OH)D_3 binding but that this role is probably a somewhat nonspecific one.

Modification of arginine residues with cyclohexanedione produced a rather different result. DBP/Gc protein contains $\sim$14 arginine residues per molecule of protein (Imawari et al., 1976; Svasti et al., 1979). Six of the arginine residues were found to be readily accessible to the modifying reagent, whereas the remaining arginine residues were much less so. Modification of the readily accessible six arginine residues had almost no effect on the binding activity of the protein for 25(OH)D_3. Further modification, however, of the less accessible arginine residues resulted in a progressive loss of binding activity, with almost complete loss of binding activity for 25(OH)D_3 being observed when 11 to 12 arginine residues were modified. The loss of binding activity was fully reversible upon regeneration of intact arginine residues with hydroxylamine. The results suggest that almost half of the arginine residues of DBP play no role in those aspects of DBP structure that are related to 25(OH)D_3 binding but that the remaining arginine residues play a significant structural role in this respect.

DBP/Gc protein contains approximately 15 to 16 tyrosine residues per molecule protein (Imawari et al., 1976). Iodination of DBP at a low level ($\leq$1 atom iodine per molecule) did not affect the binding activity of DBP for 25(OH)D_3. More extensive iodination, however, between 1 and 5 atoms of iodine incorporated per molecule of DBP, resulted in a progressive loss of more than 90% of its 25(OH)D_3 binding activity. Thus, the protein–ligand interaction appeared to be quite sensitive to iodination at levels beyond 1 atom of iodine per molecule of protein.

None of these modifications (of lysine, arginine, or tyrosine residues) had any significant effect upon DBP immunoreactivity. In contrast, immunoreactivity was rapidly lost upon reductive alkylation of disulfide bonds.

DBP contains approximately 20 to 21 half-cystine residues per molecule (Imwari et al., 1976; Svasti et al., 1979) and less than 1 accessible monothiol. In the absence of guanidine the disulfide bonds of DBP were highly resistant to reduction. Prolonged reduction of DBP in the presence of 6 M guanidine, however, resulted in the appearance of 15 assayable monothiol groups per molecule of protein. Thus, even in the presence of guanidine not all of the disulfide bonds apparently underwent reduction.

Reductive alkylation of disulfide bonds profoundly decreased both the 25(OH)D_3 binding activity and the immunoreactivity of DBP. When the protein was reacted with an equimolar amount of DTT and alkylated with monoiodoacetic acid, the resulting preparation (containing 1.1 carboxymethyl group per molecule) retained only 12% of its initial binding activity and 35% of its immunoreactivity. When the protein was alkylated in the presence of a 2.8-fold molar excess of DTT, 5 carboxymethyl groups were incorporated per molecule, and both binding activity and immunoreactivity were completely lost.

These results do not necessarily mean that DBP contains one or two specific disulfide bonds that are very susceptible to reduction with DTT and that are essential for ligand binding and immunoreactivity. It is known that reduction of disulfide bonds catalyzed by a small amount of reducing agent causes interchange of disulfide bonds (Wall, 1971). The DBP preparations alkylated in the presence of small amounts of DTT showed several bands on disc gel electrophoresis, suggesting that a good deal of disulfide bond interchange had occurred. The results do, however, suggest that a limited number of disulfide bonds play a major and critical role in maintaining the stable three-dimensional structure of DBP that is required for its 25(OH)D_3 binding activity and for its interaction with the anti-DBP antibodies.

In the presence of 6 M guanidine, DBP displayed virtually no binding affinity for 25(OH)D_3. The effects of guanidine on the association of DBP with its ligand were, however, completely reversible. These observations further demonstrate the structural stability of the DBP molecule provided that its disulfide bonds remain intact.

The studies reported here provide descriptive information about the effects of various protein modification procedures on the interaction of DBP with 25(OH)D_3. Similar studies with plasma lipoproteins have shown that the arginine and lysine residues of apolipoproteins B and E are importantly involved in the interaction of these apoproteins with cell surface receptors (Mahley et al., 1977, 1979; Weisgraber et al., 1978). Protein modification procedures have also been used to explore the binding of corticosteroids to their plasma binding protein, transcortin. These studies have examined the effects of urea and other variables (Chan & Slaunwhite, 1977) and have explored the structure of the corticosteroid binding site with affinity labeling reagents (Khan & Rosner, 1977; Le Gaillard & Dautrevaux, 1978). In a recent study with transcortin, electron spin resonance experiments were conducted with spin-labeled corticosteroid analogues, and studies were conducted with thiol reagents, to provide information about binding site topography (Defaye et al., 1980).

The two major Gc alleles (Gc^1 and Gc^2) direct the production of Gc isoproteins (Gc 1 and Gc 2) which have been reported recently to show differences in primary structure involving a very small number of amino acids (Svasti et al., 1979). Gc 1 protein exists in two major forms, Gc 1_{fast} and Gc 1_{slow}; Gc 2 protein is found as a major form (Gc 2_{slow}) and a minor component (Gc 2_{fast}) (Svasti et al., 1979; Svasti & Bowman, 1978). The three major forms of DBP/Gc protein were all found to have both amino- and carboxy-terminal leucine residues and to exhibit identical amino-terminal sequences of 20 residues (Svasti et al., 1979). DBP/Gc protein contains a small amount of carbohydrate, and it has been reported that the difference between the two forms of Gc 1 protein is postranslational in nature, involving carbohydrate dissimilarities (Svasti et al., 1979; Svasti & Bowman, 1978; Cleve & Patutschnick, 1979).

We have previously reported that the different genetic variants of DBP/Gc protein all appeared to have similar binding properties for the major vitamin D related sterols (Kawakami et al., 1979). Accordingly, although the studies reported here were carried out with a preparation of apo-DBP consisting largely of the Gc 1 isoform, it is likely that the

findings would apply to the Gc 2 isoform as well.

The extinction coefficient ($E^{1\%}_{1cm}$) at 280 nm observed here (5.21) was substantially lower than the value of 9.1 previously reported (Haddad & Walgate, 1976a; Imawari et al., 1976). In order to explore factors that might contribute to this difference, we compared the absorption spectrum of apo-DBP with that of the DBP–25(OH)D_3 complex, since in our earlier report (Imawari et al., 1976) we apparently isolated and studied holo-DBP. The observed $E^{1\%}_{1cm}$ at 280 nm for the DBP–25(OH)D_3 complex was, however, only 6.2. At the present time, the reasons for the discrepancy between the present and the earlier reported values for the extinction coefficient remain unexplained.

Finally, the spectral studies with apo-DBP and the DBP–25(OH)D_3 complex demonstrated that 25(OH)D_3 bound to DBP displayed a considerably reduced absorbance throughout its absorption spectrum, compared to 25(OH)D_3 alone in solution in ethanol. In this regard, DBP differs from the vitamin A transport protein, retinol-binding protein, since retinol bound to its transport protein displays an absorption spectrum and a molar extinction that are virtually identical with those of retinol alone in solution in organic solvents (Kanai et al., 1968; Goodman & Raz, 1972).

Acknowledgments

We thank Dr. John Edgar Smith for advice and discussions, Dr. Stephen Birken for the amino acid analyses, and R. Brown for expert technical assistance.

References

Bouillon, R., Van Baelen, H., Rombauts, W., & De Moor, P. (1976) *Eur. J. Biochem. 66*, 285–291.

Bouillon, R., Van Baelen, H., & De Moor, P. (1977) *J. Clin. Endocrinol. Metab. 45*, 225–231.

Chan, D. W., & Slaunwhite, W. R., Jr. (1977) *Arch. Biochem. Biophys. 182*, 437–442.

Cleland, W. W. (1964) *Biochemistry 3*, 480–482.

Cleve, H. (1973) *Isr. J. Med. Sci. 9*, 1133–1146.

Cleve, H., & Patutschnick, W. (1979) *Hum. Genet. 47*, 193–198.

Daiger, S. P., & Cavalli-Sforza, L. L. (1977) *Am. J. Hum. Genet. 29*, 593–604.

Daiger, S. P., Schanfield, M. S., & Cavalli-Sforza, L. L. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2076–2080.

Defaye, G., Basset, M., Monnier, N., & Chambaz, E. M. (1980) *Biochim. Biophys. Acta, 623*, 280–294.

De La Llosa, P., Durosay, M., Tertrin-Clary, C., & Jutisz, M. (1974) *Biochim. Biophys. Acta 342*, 97–104.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Giblett, E. R. (1969) in *Genetic Markers in Human Blood*, pp 160–175, F. A. Davis, Philadelphia, PA.

Goodman, DeW., & Raz, A. (1972) *J. Lipid Res. 13*, 338–347.

Haddad, J. G., Jr., & Walgate, J. (1976a) *J. Biol. Chem. 251*, 4803–4809.

Haddad, J. G., Jr., & Walgate, J. (1976b) *J. Clin. Invest. 58*, 1217–1222.

Hirs, C. H. W. (1967) *Methods Enzymol. 11*, 199–203.

Hirshfeld, J. (1962) *Prog. Allergy 6*, 155–186.

Imawari, M., & Goodman, DeW. S. (1977) *J. Clin. Invest. 59*, 432–442.

Imawari, M., Kida, K., & Goodman, DeW. S. (1976) *J. Clin. Invest. 58*, 514–523.

Kanai, M., Raz, A., & Goodman, DeW. S. (1968) *J. Clin. Invest. 47*, 2025–2044.

Kawakami, M., Imawari, M., & Goodman, DeW. S. (1979) *Biochem. J. 179*, 413–423.

Khan, M. S., & Rosner, W. (1977) *J. Biol. Chem. 252*, 1895–1900.

Le Gaillard, F., & Dautrevaux, M. (1978) *FEBS Lett. 94*, 63–67.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Mahley, R. W., Innerarity, T. L., Pitas, R. E., Weisgraber, K. H., Brown, J. H., & Gross, E. (1977) *J. Biol. Chem. 252*, 7279–7287.

Mahley, R. W., Weisgraber, K. H., & Innerarity, T. L. (1979) *Biochim. Biophys. Acta 575*, 81–91.

McFarlane, A. S. (1958) *Nature (London) 182*, 53.

Means, G. E., & Fenney, R. E. (1968) *Biochemistry 7*, 2192–2201.

Patthy, L., & Smith, E. L. (1975) *J. Biol. Chem. 250*, 557–564.

Putman, F. W. (1977) *The Plasma Proteins*, Vol. 3, pp 333–357, Academic Press, New York.

Robyt, J. F., Ackerman, R. J., & Chittenden, C. G. (1977) *Arch. Biochem. Biophys. 147*, 262–269.

Spackman, D. H., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30*, 1190–1206.

Svasti, J., & Bowman, B. H. (1978) *J. Biol. Chem. 253*, 4188–4194.

Svasti, J., Kurosky, A., Bennett, A., & Bowman, B. H. (1979) *Biochemistry 18*, 1611–1617.

Wall, J. S. (1971) *J. Agric. Food Chem. 19*, 619–625.

Weisgraber, K. H., Innerarity, T. L., & Mahley, R. W. (1978) *J. Biol. Chem. 253*, 9053–9062.

Zahler, W. L., & Cleland, W. W. (1968) *J. Biol. Chem. 243*, 716–719.