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Dye-Ligand Interactions with 1,25-Dihydroxyvitamin D_3-Receptor Complexes from Chicken Intestine

WILLIAM S. MELLON

School of Pharmacy, University of Wisconsin-Madison, Madison, Wisconsin 53706

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SUMMARY

A number of dye-ligand adsorbents have been examined for purifying and characterizing 1,25-dihydroxyvitamin D_3-receptor complexes from intestines of vitamin D_3-deficient chickens. In particular, several triazinyl dyes—Cibacron blue F3GA, Procion red HE3B, and Green A dye, immobilized to agarose via an ether linkage—retain specifically bound 1,25-dihydroxyvitamin D_3-receptor complexes formed at 0–4° which are eluted at high salt concentrations. Moreover, receptor binding to these dye-ligand matrices occurs in the presence and absence of sterol. At least for Cibacron blue, the strength of receptor binding depends critically on the method of dye coupling to matrix. The concentration of KCl required for elution of receptor from the triazine ether-linked matrix is > coupling through the amine of the anthraquinone via a 10-atom spacer arm ≅ to coupling through the amine of the anthraquinone via an isourea bond > Cibacron blue dextran. Data are presented which demonstrate that sterol-receptor complexes formed at 25° have reduced affinity for dye-ligands when compared with sterol-receptor complexes formed at 0–4°. It is suggested that this finding is related to proteolytic alterations of the receptor, since limited digestion with trypsin can mimic this phenomenon and several protease inhibitors can reduce the thermal-induced alterations. Biospecific elution of receptor is demonstrated using synthetic polyribonucleotides. Preference for polyguanylic and polyinosinic acid is observed over several other polyribonucleotides and mononucleotides. The data in this study, viewed collectively, suggest that there is a specific interaction between the polynucleotide domain of the 1,25-dihydroxyvitamin D_3-receptor and several triazinyl dye-ligands. It is concluded that these dye-ligands should prove to be of considerable interest for facile chromatography to purify and characterize this receptor.

INTRODUCTION

Aromatic dye molecules tend to bind to globular proteins preferentially in areas overlapping the binding sites for substrates, coenzymes, and prosthetic groups (1). The anthraquinone reactive dye, CB,[1] has been utilized as a useful probe with a variety of applications. When immobilized, CB has been employed to purify nucleotide-requiring enzymes (2–6), mammalian interferons (7–9), and the chicken intestinal receptors for $1,25(OH)_2D_3$ (10). In addition to these specific references, dyes have been used to isolate a wide variety of proteins, and extensive lists of such applications have been compiled (11, 12).

The elution of bound proteins from dye-substituted resins has been carried out employing increasing ionic strength (3, 7, 8), hydrophobic solvents (2, 7, 8), and in some cases by specific substrates (3, 6, 9, 13). The latter procedure of biospecific elution is an example of true affinity chromatography. Interestingly, the degree to which proteins demonstrate affinity for immobilized dye-ligands depends upon precise orientation and availability of the dye. Beissner and Rudolph (3) previously have shown that, although the entire CB molecule is not required for biospecific binding and elution, the 1-amino-anthraquinone-2-sulfonic acid moiety is critical for binding selected enzymes, a kinase and several dehydrogenases. Moreover, several mammalian interferons display different chromatographic behavior on CB-substituted matrices (7, 8). Generally, CB coupled to agarose through the triazine ring via an ether linkage has the strongest

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[1] The abbreviations used are: CB, Cibacron blue F3GA; PR, Procion red HE3B; GA, Green A dye; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; PPO 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazoyl)]benzene; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl; TED, 50 mM Tris-HCl 1.5 mM EDTA/0.5 mM dithiothreitol (pH 7.5); TMD, 50 mM Tris-HCl/0.5 mM $MgCl_2$/0.5 mM dithiothreitol; TEDK-0.03 or −0.15, or TMDK-0.03 or −0.15, etc., TED or TMD buffer with 0.03 M KCl, 0.15 M KCl, etc.

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binding for interferons. Blue dextran-agarose displays intermediate binding properties for several mammalian interferons (8), whereas the chromophore immobilized on cyanogen bromide-activated cross-linked agarose by the amino group of the anthraquinone results in the weakest strength of binding (7, 8). Thus, free amino groups on the CB molecules appear to be of primary importance in binding interferons.

The usefulness of dye-ligand chromatography for purification purposes and as a means of gaining information about the topology of proteins has led us to examine whether the chicken intestinal $1,25(OH)_2D_3$ receptor could interact with several dye structures. This paper describes the ability of several immobilized triazinyl textile dyes to bind $1,25(OH)_2D_3$ receptors. Conditions are established for the biospecific elution of receptor complexes with polyribonucleotides. In addition, results are presented which demonstrate the utility of these immobilized dye-ligands to differentiate between native and proteolytic digested forms of the $1,25(OH)_2D_3$ receptor.

MATERIALS AND METHODS

Animals. One-day-old white Leghorn cockerels (Northern Hatcheries, Beaver Dam, Wisc.) were fed a vitamin D-deficient soy protein diet (Teklad Test diet, TD 80146, Madison, Wisc.) and received water ad libitum for 3–4 weeks. All birds were maintained in a vivarium at 25–26° on an alternating 12-hr light/12-hr dark cycle using incandescent lighting.

Chemicals. Nonradioactive $1,25(OH)_2D_3$ was a generous gift from Dr. M. Uskokovic, of the Hoffman-La Roche Company (Nutley, N. J.). Determination of purity and concentration of nonradioactive $1,25(OH)_2D_3$ was achieved by ultraviolet absorption spectroscopy using an extinction coefficient (ϵ = 264) of 18,200 M^{-1} cm^{-1}. $1,25(OH)_2[26,27\text{-}^3H]D_3$ (148–160 Ci/mmole) was obtained from Amersham Corporation (Arlington Heights, Ill.). Other chemicals used and their sources are as follows: dithiothreitol, Calbiochem (La Jolla, Calif.); Affi-Gel 202 (17.3 μmoles of COOH per milliliter, Bio-Rad Laboratories (Richmond, Calif.); Sepharose 4B, Pharmacia (Piscataway, N. J.); Urea (ultrapure), Schwarz/Mann (Orangeburg, N. Y.); ethylene glycol (certified), Fisher Scientific (Fair Lawn, N. J.); CB, Pierce Chemical Company (Rockford, Ill.); octyl-agarose, Miles Laboratories, Inc. (Elkhart, Ind.); dye-ligand matrices (dyes bound to agarose via an ether linkage through the triazine ring), CB, PR, copper phthalocyanine chromophore, Orange A dye, and GA, Amicon Corporation (Lexington, Mass.); PPO, POPOP, and Triton X-100, RPI Corporation (Elk Grove Village, Ill.); and charcoal (neutralized and washed to remove the "fines"), dextran (M_r 70,000), Tris, EDTA, bovine serum albumin (Fraction V), polyadenylic acid (5′), polycytidilic acid (5′), polyguanylic acid (5′), polyinosinic acid (5′), polyuridylic acid (5′), adenosine, AMP (5′), ADP (5′), ATP (5′), NAD, NADP, heparin, poly-L-glutamic acid, blue dextran-agarose, cyanogen bromide, EDAC, trypsin (from bovine pancreas, EC 3.4.21.4), and trypsin inhibitor (from soybean), Sigma Chemical Company (St. Louis, Mo.). All other reagents were of analytical grade.

Preparation of $1,25(OH)_2D_3$ binding proteins. Chickens were deprived of food for 16–20 hr after which time they were killed by cervical dislocation and the duodenal loop was freed of pancreas and excised. All procedures were carried out between 0 and 4°. Mucosa was scraped free of serosa and washed three times in several volumes (w/v) of buffer TEDK-0.15. Washed mucosa was homogenized in 5 volumes of TEDK-0.30 buffer or TMDK-0.30 buffer as described previously (14). The cytoplasmic fraction, containing $1,25\text{-}(OH)_2D_3$ binding proteins, was prepared by centrifuging the homogenate at 78,000 × *g* for 90 min in a Beckman L5-50B ultracentrifuge using a Type 30 rotor (Beckman Instruments, Palo Alto, Calif.). Cytosol was frozen in an isopropanol/dry ice bath, followed by lyophilization (FTS Systems Inc., Stoneridge, N. Y.), and stored at −70° until use.

Nuclear $1,25(OH)_2D_3$ binding proteins were obtained from isolated intestinal nuclei prepared by the hexylene glycol procedure described previously (14). After the final centrifugation through 2.0 M sucrose, the pelleted nuclei were rinsed once with TMDK-0.03 buffer. The isolated nuclei were subjected to TEDK-0.6 buffer at 0–4° for 45 min with vortexing at 10 to 15-min intervals. The nuclear chromatin extracts were centrifuged in an ultracentrifuge using a Type Ti 50 rotor (Beckman Instruments) at 105,000 × *g* for 45 min. The clear supernatant was utilized for the source of nuclear $1,25(OH)_2D_3$ binding proteins. Protein concentration was determined by the method of Bradford (15), using crystalline bovine serum albumin as a standard.

Receptor labeling. Cytosol or nuclear extracts were incubated with 2.0 nM $1,25(OH)_2[26,27\text{-}^3H]D_3$ (148–160 Ci/mmole) at 0–4° or 25° in TEDK-0.3 buffer. Specific times are indicated in the figure legends. In some cases, parallel incubations were conducted in the presence of a 200-fold excess of nonradioactive $1,25(OH)_2D_3$ to measure nonspecific binding. Radioactive and nonradioactive sterols were added in cold absolute ethanol (2.5–5.0%, v/v). After saturation of receptor with sterol had occurred, unbound sterol was removed by dextran-coated charcoal adsorption [0.05% and 0.5%, (w/v) respectively)]. The $1,25(OH)_2[^3H]D_3$-receptor complexes were diluted with TED buffer to reduce the KCl concentration to an appropriate level prior to chromatography.

Chromatographic procedures. Receptor labeled with $1,25(OH)_2[^3H]D_3$ was applied to dye-ligand columns (1.1 × 3.7 cm) (see Fig. 1) equilibrated with TEDK-0.15 at 0–4° (unless otherwise stated in the figure legends). Chromatography was initiated by washing the column with equilibrating buffer followed by a linear concentration gradient of KCl as specified in each figure legend. In some cases, the column was developed further with a mixture of ethylene glycol/TEDK-0.15 (3:2, v/v). The columns were developed at a flow rate of 7.0–12.5 ml/cm^2 per hour. Fractions were collected, and 0.5 ml from each fraction was routinely assayed for radioactivity. An aliquot (4.0 ml) of scintillation fluid consisting of 1.32 liters of Triton X-100, 16 g of PPO, and 0.2 g of POPOP per 4 liters of toluene was added to each vial. The radioactivity was determined by liquid scintillation spectrometry in a Packard Tri-Carb spectrometer (Prias PLD) with an efficiency for tritium of 45%. Quench correction was determined by the use of automatic external standardization. Salt gradients were measured by conductivity. Recovery of radioactivity from dye-ligand columns was generally 70–75% and >95% when additionally stripped with ethylene glycol (60%).

Coupling of CB to Affi-Gel 202. An Affi-Gel 202 slurry (10 ml) was washed with 10 volumes of deionized distilled water followed by two washes of 10 volumes each of 0.01 M MOPS buffer (pH 7.4) containing 0.15 M KCl. After filtration, the resin was resuspended in 10 ml of the above buffer. EDAC [165.8 mg dissolved in 2.0 ml of 0.01 M MOPS (pH 7.4)/0.15 M KCl] was added to the resin. Subsequently, 156 mg of CB were dissolved in 3.0 ml of the above buffer and mixed with the resin and carbodiimide. The coupling was allowed to proceed for 20 hr at 0–4° with constant gentle stirring. The resin, coupled through the primary amine of the anthraquinone portion of the CB molecule via a 10-atom spacer arm, was extensively washed with deionized distilled water (40 volumes), 2.0 M KCl (40 volumes), 8 M urea (40 volumes), and finally with TEDK-0.15 (pH 7.5) (50 volumes).

Coupling of CB to cyanogen bromide-activated Sepharose 4B. Sepharose 4B was washed with 20 volumes of deionized distilled water, filtered, and resuspended in 1 volume of water. To this was added 1 volume of 2 M sodium carbonate, which was mixed by stirring slowly. Activation with cyanogen bromide was carried out as described by March *et al.* (16). After activation, the slurry was poured onto a sintered-glass funnel and washed with 20 volumes each of 0.1 M sodium bicarbonate (pH 9.5) and 0.4 M sodium bicarbonate (pH 9.5). The compact cake was transferred to a flask containing 100 mg of CB and 23.5 ml of 0.4 M sodium bicarbonate (pH 9.5) and stirred continuously at 0–4° for 20 hr. The coupled primary amine of the CB to the cyanogen bromide-activated Sepharose via an isourea bond was treated with

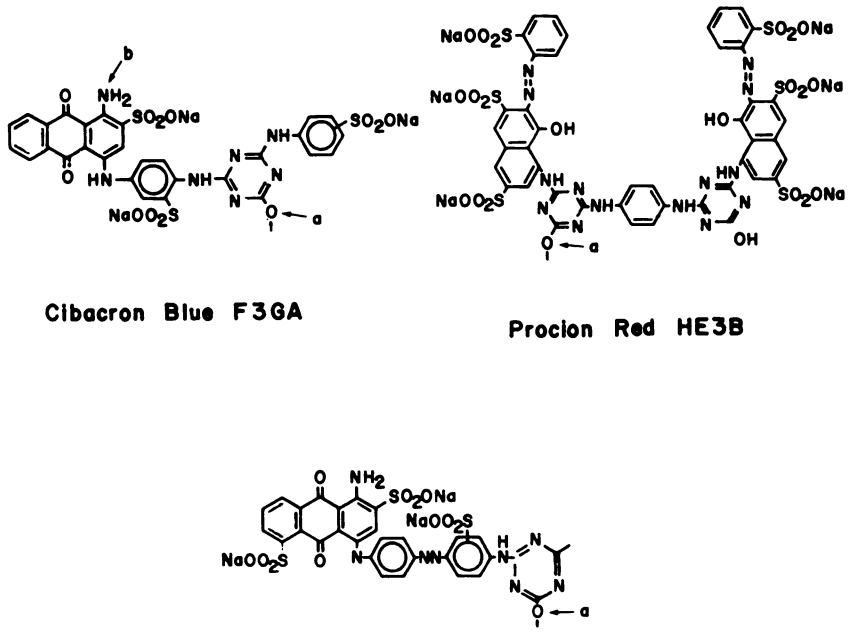

FIG. 1. *Structures of the dye-ligands CB and PR and the partial structure of GA used in the study*

The dyes were immobilized to agarose at the positions indicated (*a*) by substitution at the triazine ring via an ether linkage to agarose (CB-agarose, PR-agarose, and GA-agarose), (*b*) by a carbodiimide-catalyzed reaction with the primary amine and 10-atom carboxyl spacer arm on agarose (CB-Affi-Gel 202), or via interaction with cyanogen bromide-activated agarose (CB-NH-agarose). In some cases, CB was coupled to the dextran moiety by the triazine linkage; the complex in turn coupled to cyanogen bromide-activated agarose.

successive washes of 0.1 M sodium acetate (pH 4.0) containing 0.5 M NaCl (40 volumes), 8 M urea (40 volumes), 0.1 M sodium bicarbonate (pH 10) containing 0.5 M NaCl, and finally with 75 volumes of TEDK-0.15 (pH 7.5).

Determination of CB substitution. The amount of covalently bound CB dye present in preparations of cyanogen-activated Sepharose 4B and Affi-Gel 202 was determined by the method of Chambers (17). Briefly stated, the method involves hydrolysis of the immobilized dye-resin in 6 M hydrochloric acid followed by spectrophotometric determination of the dye released at 515 nm.

RESULTS

We have utilized a variety of immobilized dye-ligands to screen for potentially useful ligands capable of purifying and/or characterizing $1,25(OH)_2D_3$ receptors. Of those tested, only three dye-ligands were capable of retaining specifically labeled $1,25(OH)_2D_3$ chick intestinal cytosol. These dye-ligands were immobilized to agarose via an ether linkage through the triazine ring structure (Fig. 1). As can be seen in Fig. 2, $1,25(OH)_2[^3H]D_3$-

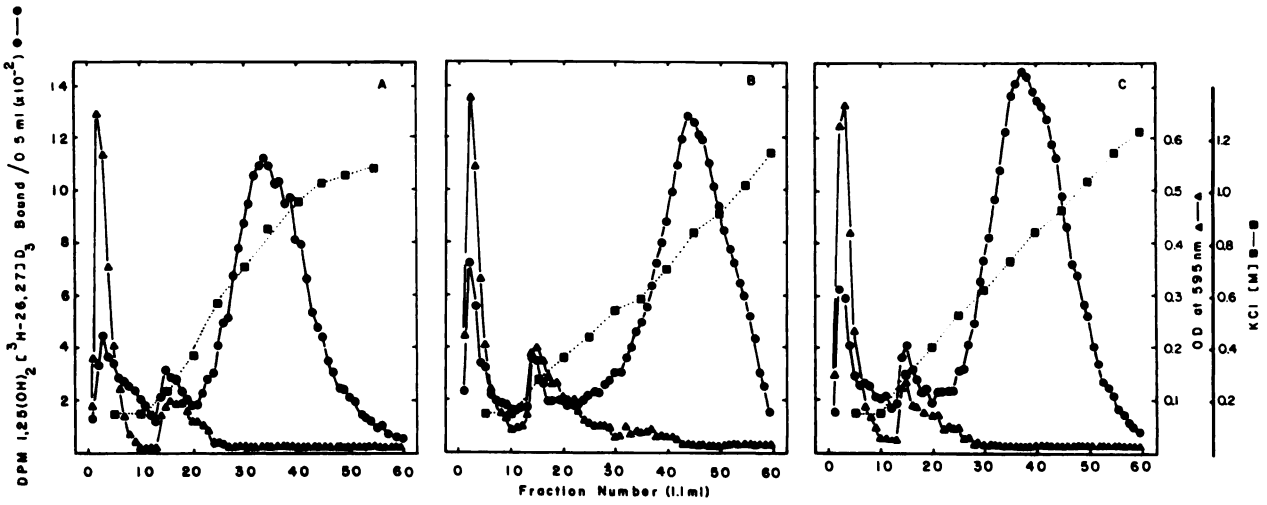

FIG. 2. *Dye-ligand chromatography of 1,25-dihydroxyvitamin D_3-receptor complexes of chick intestinal cytosol*

Aliquots (0.3 ml) of labeled cytosol (15 mg of protein per milliliter were applied to dye-ligand-agarose columns (2.0-ml bed volume) equilibrated with TEDK-0.15 buffer. A, CB (1.85 mg of dye per milliliter of gel bed); B, PR (1.97 mg of dye per milliliter of gel bed); C, GA (1.84 mg of dye per milliliter of gel bed). Chromatography was initiated by washing the columns with TEDK-0.15 buffer, followed by a linear KCl gradient elution (0.15–1.50 M). Fractions (1.1 ml) were collected and 0.5-ml aliquots were removed to determine the radioactivity.

receptor complexes formed at 0–4° are retained by several immobilized dyes: CB, PR, and GA in TEDK-0.15 buffer (pH 7.5). Elution with a salt gradient (0.15–1.50 M KCl) resulted in a peak of specifically bound radioactivity eluting maximally at 0.8 M KCl. Subsequently, we have observed tighter binding of the receptor to other batches of dye-ligand resins (particularly CB) such that labeled receptor elutes at 1.0–1.2 M KCl. The elution of protein from these columns occurs primarily in the drop-through fractions, whereas minimal amounts are eluted concomitantly with labeled receptor. By utilizing analytical immobilized dye-ligand columns, we have obtained 8- to 10-fold purifications of $1,25(OH)_2[^3H]D_3$-receptor complexes from intestines of vitamin D-deficient chicks with yields of 65–75% for specifically bound $1,25(OH)_2D_3$. The affinity of chick intestinal $1,25(OH)_2D_3$ receptors for these dye-ligands does not depend on the sterol's being bound to the receptor. The majority of receptor is eluted at a high salt concentration whether chromatographed in the presence or absence of $1,25(OH)_2D_3$ (data not shown).

When chick intestinal cytosol is labeled at 25° and applied to dye-ligand columns, several peaks of bound radioactivity elute. Incubation periods of 45 min at 25° result in the elution of bound radioactivity at 0.4 M KCl and 0.8 M KCl (Fig. 3A), whereas longer times (90 min) result in almost complete loss of the bound radioactivity eluting at 0.8 M KCl (Fig. 3B). Also, receptor labeled at 25° behaves similarly when chromatographed on PR- and GA-agarose (data not shown). The bound radioactivity that elutes at a lower salt concentration (0.4 M KCl) is still specifically bound, as it is displaceable by an excess of nonradioactive $1,25(OH)_2D_3$. In addition, radioactivity from the 0.4 M KCl peak migrates on sucrose density gradients with sedimentation properties similar to those of cytosol, which is labeled at 0–4° (data not shown). Interestingly, cytosol that is labeled at 0–4° with $1,25(OH)_2[^3H]D_3$ and then warmed to 25° for 45 min also results in the appearance of a peak of radioactivity eluting at 0.4 M KCl (data not shown).

Receptor that is isolated from intact intestinal nuclei also is sensitive to a temperature-induced change in the elution profile on immobilized dye-ligands. Similar to the receptor isolated from the cytoplasm, nuclear receptor predominantly elutes from a CB-agarose column at 0.8 M KCl when $1,25(OH)_2[^3H]D_3$-receptor complexes are formed at 0–4°. Further incubation of $1,25(OH)_2[^3H]D_3$-receptor complexes at 25° for 1.0 hr results in elution of specifically bound radioactivity at 0.4 M KCl (data not shown).

To assess whether the changes in the salt elution profile on CB-agarose with cytosol labeled at 25° could be the result of proteolytic activity, cytosol was labeled at 0–4° with $1,25(OH)_2[^3H]D_3$ until saturation. The $1,25(OH)_2[^3H]D_3$-receptor complexes were then subjected to partial tryptic digestion (10 μg of trypsin per milligram of protein) at 0–4° for 30 min. When applied to CB-agarose and chromatographed with TEDK-0.15 followed by a buffered KCl gradient (0.15–2.0 M), bound radioactivity elutes both at 0.4–0.5 M KCl and at high

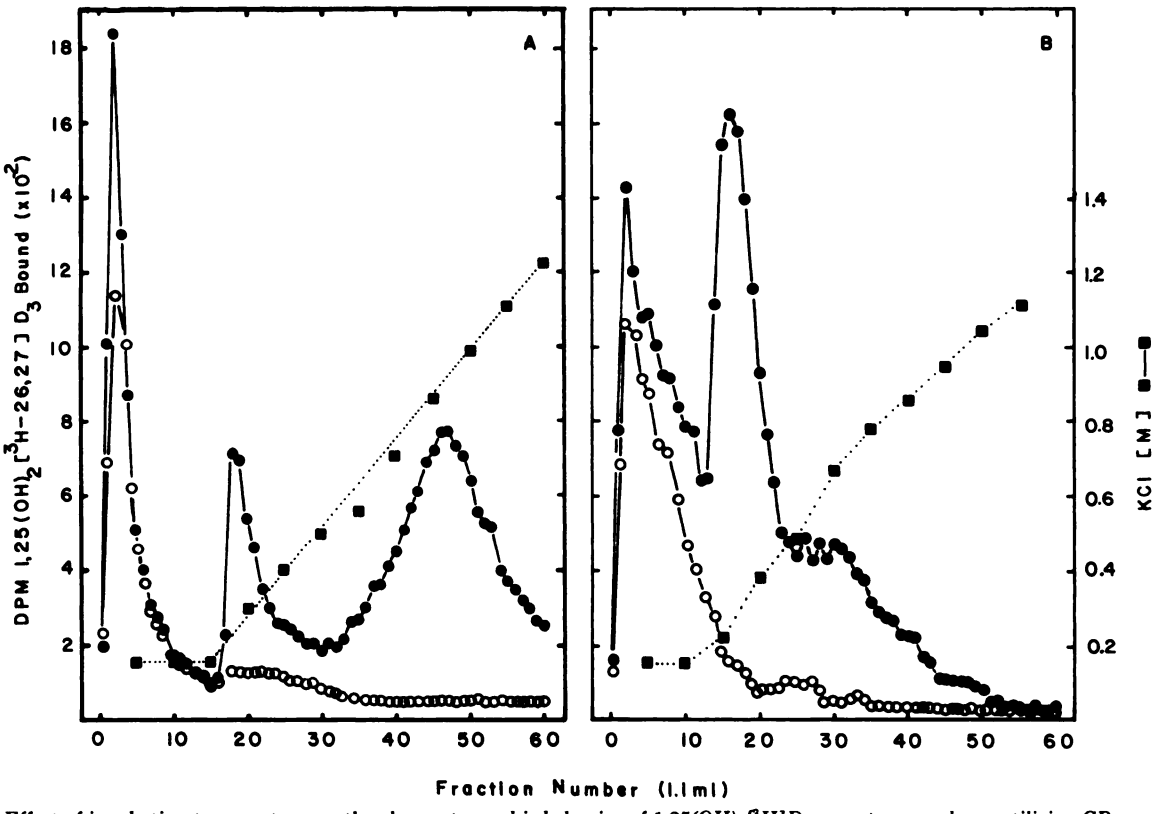

FIG. 3. *Effect of incubation temperature on the chromatographic behavior of $1,25(OH)_2[^3H]D_3$-receptor complexes utilizing CB-agarose*
Chick intestinal cytosol (15 mg of protein per milliliter was incubated with 2.0 nM $1,25(OH)_2[^3H\text{-}26,27]D_3$ (160 Ci/mmole) for (A) 45 min or (B) 90 min at 25°. Chromatography was conducted as described in Fig. 2.

salt (1.1 M KCl) (data not shown). Since exogenous addition of trypsin to labeled cytosol produced a chromatographic pattern on CB-agarose similar to that of 25°-labeled cytosol, trypsin inhibitor (20 μg/mg of protein) was added to cytosol prior to warming to determine whether it would retard the thermal-induced changes. Likewise, the use of other proteolytic inhibitors (phenylmethylsulfonyl fluoride, 500 μM; aprotinin, 15 trypsin inhibitor units/2.5 ml of cytosol) also retarded the thermal-induced change (data not shown).

Since recovery of total bound radioactivity from analytical dye columns was on the order of 65–75% (e.g., see Figs. 2 and 3), several ways were investigated to improve recovery. Among the parameters tested, including pH, ionic strength, ethylene glycol, sample size, flow rate, and column dimensions, only ethylene glycol improved the recovery of bound radioactivity. Therefore, we began to strip the columns with ethylene glycol/TEDK-0.15 (3:2, v/v) after completion of the salt gradient. This resulted in an increase in the recovery of total radioactivity to greater than 90%. It should be noted that the radioactivity eluting with the ethylene glycol is not competed for by a 200-fold excess of nonradioactive $1,25(OH)_2D_3$, whereas greater than 95% of the radioactivity eluting under high-salt conditions is specifically bound.

The method of immobilizing dye-ligands to a solid support can affect the affinity of $1,25(OH)_2D_3$ receptors for these dyes. In contrast to the requirement of high salt to elute receptor from CB-agarose, elution from blue dextran-agarose occurred at a much lower salt concentration (0.3 M KCl) (Fig. 4). To investigate further whether precise orientation of the CB molecule could affect receptor binding, we coupled CB via its amine on the anthraquinone to agarose by two methods. When labeled cytosol was applied on a CB-Affi Gel 202 column, retention of bound radioactivity occurred and was eluted maximally at 0.47 M KCl. Likewise, labeled cytosol that bound to CB-NH-agarose was eluted maximally at a KCl concentration of 0.4 M (data not shown).

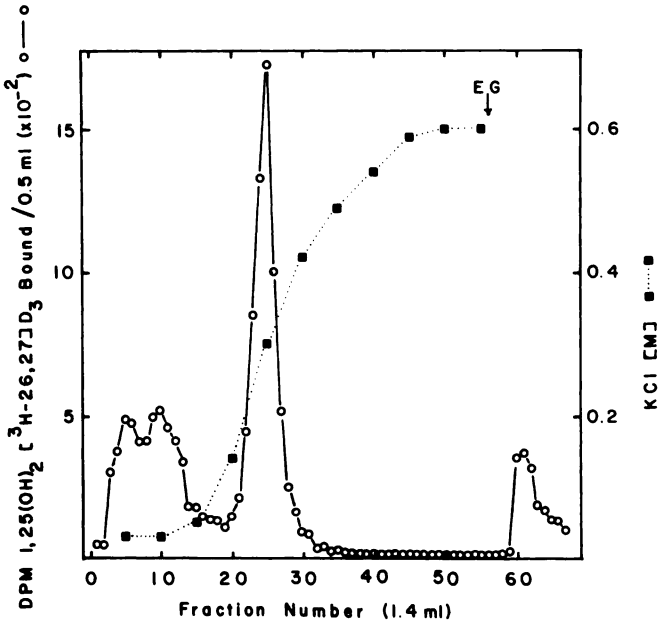

FIG. 4. *Blue dextran-agarose chromatography of $1,25(OH)_2[^3H]D_3$-receptor complexes of chick intestinal cytosol*

A 0.2-ml aliquot (diluted to 0.03 M KCl) of labeled cytosol was applied to a blue dextran-agarose column (1.1 × 3.7 cm; 2.1 mg of blue dextran per milliliter of packed gel). Chromatography was initiated by washing the column with TEDK-0.03 buffer, followed by a linear KCl gradient elution (0.03–1.00 M). Finally, the column was eluted with ethylene glycol-TED buffer (3:2) (*EG*). Fractions (1.4 ml) were collected and 0.5-ml aliquots were removed to determine the radioactivity.

TABLE 1

Desorption by nucleotides of $1,25(OH)_2[^3H]D_3$-labeled chick intestinal cytosol bound to CB-agarose or blue dextran-agarose

Aliquots of labeled cytosol were diluted to 0.03 M KCl and applied to CB-agarose (0.28 mg of CB per milliliter of gel bed) (1.1 × 3.7 cm) or blue dextran-agarose (2.1 mg of CB per milliliter of gel bed) (1.1 × 3.7 cm) and eluted with 5 column volumes of TMDK-0.03 buffer (pH 7.5), 3 bed volumes of a given nucleotide, and finally with TMDK-1.5 for CB-agarose or TMDK-0.5 for blue dextran-agarose. Recoveries of total radioactivity, including an ethylene glycol strip, were all >90%. Aliquots (0.5 ml) from each 1.5-ml fraction were assayed for radioactivity. Shown are the respective percentages of eluted specifically bound radioactivity obtained with each nucleotide tested. Values have been corrected so that the percentage eluted with the nucleotide plus the percentage of desorption with TEDK-1.5 equals 100.

Eluant	Concentration	% Eluted from column	
		CB-agarose	Blue dextran-agarose
	mM		
NAD	100	3.6	3.8
NADP	100	5.4	5.2
ATP[a]	100	5.9	4.9
Poly-L-glutamate[b]	100	—	3.5
Heparin[c]	100	—	5.2
	mg/ml		
Poly(A)	0.2	1.3	1.7
Poly(C)	0.2	1.9	4.5
Poly(G)	0.2	50.8, 46.1[d]	79.9, 66.1[d]
Poly(I)	0.2	16.0, 19.1[d]	81.1
Poly(U)	0.2	9.2	7.5

[a] Other analogues, including adenosine, AMP, and ADP, resulted in similar percentages.

[b] Based on molecular weight of L-glutamic acid.

[c] Based on molecular weight of a dissaccharide unit.

[d] Results of two experiments.

To examine whether $1,25(OH)_2[^3H]D_3$-receptor complexes could be eluted from immobilized dye-ligands biospecifically, rather than nonspecifically using KCl, we tested a number of nucleotides. Results given in Table 1 show that a portion of the receptor bound to blue dextran-agarose or CB-agarose (linked via the triazine ring) can be eluted by solutions of nucleotides in TMDK-0.03 buffer. The degree of desorption obtained depended on the nature of the nucleotide. Poly(G) and poly(I) completed efficiently with blue dextran, resulting in 66–81% elution of specifically labeled receptor; desorption from CB-agarose was less pronounced, with only 16–50% being eluted. Also, poly(G) was capable of causing significant desorption (60%) of $1,25(OH)_2D_3$ receptors partially trypsinized (results not shown). The profile shown in Fig. 5 reveals that desorption of receptor from blue dextran with a buffered solution of poly(G) (0.2 mg/ml) is obtained within 4 bed volumes. Other mononucleotides as well as poly(A), poly(C), and poly(U) did not elute

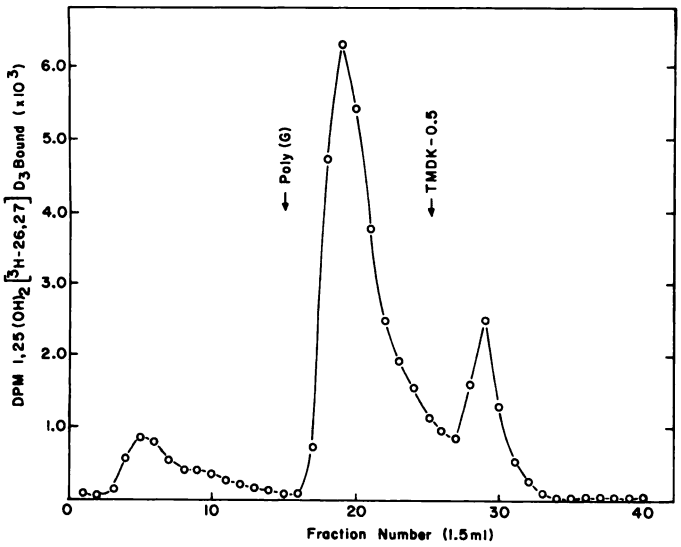

FIG. 5. *Elution of 1,25(OH)$_2$[^{3}H]D$_3$-receptor complexes from blue dextran-agarose by poly(G)*

An aliquot of labeled cytosol (6.84 mg of protein per milliliter was diluted to 0.03 M KCl and applied to blue dextran-agarose (1.1 × 3.7 cm). Chromatography was initiated by eluting with TMDK-0.03 followed by poly(G) (0.2 mg/ml of TMDK-0.03) and finally with TMDK-0.50. Aliquots (0.5 ml) were removed from each 1.5-ml fraction to quantitate radioactivity.

1,25(OH)$_2$[^{3}H]D$_3$-receptors significantly. Partial analysis of nucleotide elution from GA-agarose shows that poly(G) results in desorption of 72% of the specific receptor input, whereas poly(A), NAD, NADP, and ATP were ineffective (results not shown).

DISCUSSION

We have examined dye-ligand adsorbents as a means of purifying and characterizing 1,25(OH)$_2$D$_3$-receptor complexes. Dye-ligand chromatography may be considered a variant of affinity chromatography in which a synthetic dye replaces a normal substrate, cofactor, or another biospecific factor. In this context, CB has been used widely for purification of a number of different proteins, including dehydrogenases (2, 3, 5), and a variety of other nucleotide-requiring enzymes (6). For the present study, several triazine-linked dyes were employed to test their ability and capacity for binding 1,25(OH)$_2$D$_3$-receptor complexes. Of those that were tested, immobilized CB, PR, and GA bound 1,25(OH)$_2$D$_3$-receptor complexes isolated from vitamin D-deficient chick intestinal cytosol or nuclei.

Unlike the moderate affinity of 1,25(OH)$_2$D$_3$ reported previously for blue dextran-Sepharose (10), these receptors displayed tighter binding for the triazine-linked dyes. Similar findings for some mammalian interferons (7, 8) and several nucleotide-requiring enzymes (3) have been observed using CB coupled to agarose by several methods, the greatest strength of binding being associated with the triazine linkage. The affinity of 1,25(OH)$_2$D$_3$ receptors is decreased when the blue chromophore is linked to agarose via an isourea bond between the dye and agarose matrix or through an amide-linked 10-atom spacer arm. Thus, the anthraquinone moiety of CB with a free amino group seems to play an important role in binding 1,25(OH)$_2$D$_3$ receptors. Similar findings also have been noted for interferons (8) and several enzymes (3). The weakest binding for 1,25(OH)$_2$D$_3$ receptors occurs with blue dextran-agarose. Although the majority of CB molecules are bound to dextran by the triazine ring, there may be some steric hindrance by the dextran moiety, limiting the availability of the receptor for binding CB.

Aside from the utility of immobilized dye-ligands for purification of 1,25(OH)$_2$D$_3$ receptors, it is capable of resolving different forms of 1,25(OH)$_2$D$_3$ receptors induced by elevated temperatures. Thus, 1,25(OH)$_2$D$_3$-receptor complexes formed at 25° for various times (Fig. 3A and B) retain recognition for CB, albeit with reduced affinity (i.e., 0.4 M KCl for elution). Interestingly, limited digestion of 1,25(OH)$_2$D$_3$-labeled chick intestinal cytosol with trypsin (10 μg/mg of cytoplasmic protein) for 30 min at 0–4° produces a 1,25(OH)$_2$D$_3$ binding species which elutes from CB-agarose maximally at approximately 0.5 M KCl. Incubation with higher concentrations of trypsin (0.027–0.36 mg/mg of cytoplasmic protein) results in a progressive loss of high salt-desorbable binding and, eventually, the loss of all binding to CB-agarose.[2] This information, together with the observation that several protease inhibitors block this thermal-induced change, suggests that labeling chick intestinal cytosol at 25° may subject the receptor to proteolysis which can be detected by dye-ligand chromatography.

From the present findings, it is possible to conclude that the 1,25(OH)$_2$D$_3$ receptor is retained by immobilized dye-ligands through an interaction involving a receptor domain that is altered by proteolysis. Furthermore, elution of 1,25(OH)$_2$D$_3$ receptors specifically by poly(G) and poly(I) indicates that a significant portion of the receptor dye-ligand interaction may be occurring at a polynucleotide domain which is susceptible to heat-induced alterations. However, the ability of poly(G) to desorb partially trypsinized receptor further implies that the receptor dye-ligand interaction involves more than one domain on the receptor. Evidence for multiple sites of binding for dyes with other proteins has been reported previously (18, 19).

Several enzymes and proteins possessing a nucleotide domain have been eluted from immobilized dye-ligands with low concentrations of specific substrates (3, 6, 9, 13). More specifically, previous studies have employed successfully immobilized dye-ligands with polyribonucleotide elution to purify mouse interferon (9) and native polynucleotide phosphorylase (13). Likewise, the findings in the present study reveal that specific elution of significant amounts of receptor was achieved with two polyribonucleotides, poly(G) and poly(I) (Table 1). Thus, by utilizing the capabilities of dye-ligands to interact with the polynucleotide domain of the 1,25(OH)$_2$D$_3$ receptor, these dyes, along with polyribonucleotide elution, were tested as affinity ligands for 1,25(OH)$_2$D$_3$ receptor purification. Although we have not attempted to optimize conditions, a 20- to 30-fold purification is obtainable by chromatographing native cytosol by such a method.

The use of synthetic polynucleotides has generated

[2] W. S. Mellon, unpublished observations.

interest because of their close relationship to the naturally occurring nucleic acids. Synthetic polynucleotides are capable of assuming more than one configuration. These molecules can be found in an amorphorus, random-coil form in which there is no regular relationship between the orientation of successive nucleotides along the polymer chain (20). Indeed, under conditions of neutral pH, poly(A) and poly(C) exist as flexible chains (20, 21). In contrast, poly(I) and poly(G) form isomorphous, quadruple-stranded complexes (22, 23). The X-ray diffraction patterns of fibers of both of these polyribonucleotides have been shown to be virtually identical (22, 23). Thus the elution of $1,25(OH)_2D_3$-receptor complexes bound to immobilized CB by poly(G) and poly(I) may be related to the elongated stable helical structures which can span the polynucleotide domain on the $1,25(OH)_2D_3$ receptor. The ineffectiveness of poly(A), poly(C), and poly(U) might be a result of their randomness under conditions in which these studies were carried out. Furthermore, the inability of mononucleotides to elute bound receptor supports our theory that the $1,25(OH)_2D_3$ receptor's nucleotide domain specifically recognizes nucleotides in elongated helical conformations. The fact that the polyanions, poly-L-glutamate and heparin (Table 1), did not elute bound receptor implies that electrostatic charge alone probably is not a major factor in determining the specificity of poly(G) and poly(I). Although the triazinyl dyes have a potential for nonspecific interaction with proteins, our evidence of specific chromophore orientation, lack of elution with a nonspecific hydrophobic solvent, and selectivity for quadruple-stranded polyribonucleotides is indicative of a specific interaction between the polynucleotide domain of the $1,25(OH)_2D_3$ receptor and these dye-ligands. It is concluded that these dye-ligands should prove to be of considerable interest for facile chromatography to purify and characterize this receptor.

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Send reprint requests to: Dr. William S. Mellon, School of Pharmacy, University of Wisconsin-Madision, 425 North Charter Street, Madison, Wisc. 53706.