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Photoaffinity site-specific covalent labeling of human corticosteroid-binding globulin

(photoaffinity labeling/steroid-specific binding proteins/active-site covalent labeling)

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ABSTRACT **A method was developed for the synthesis of high-specific-activity 21-diazo-21-[*6,7*-^{3}H]deoxycorticosterone, an analog of corticosterone. This analog was used as a photoaffinity label of a high affinity steroid-binding protein, human corticosteroid-binding globulin. Based on direct binding studies and crosscompetition experiments, this diazo derivative exhibited the requisite affinity (within a factor of 1.5 times that of corticosterone) and site specificity to qualify as an affinity labeling ligand. Irradiation of corticosteroid-binding globulin with the 21-diazo derivative resulted in irreversible binding to corticosteroid-binding globulin, identified by polyacrylamide gel electrophoresis. Specificity of covalent binding to corticosteroid-binding globulin was established by competition analysis with various steroids. Irreversibility of photodependent binding was shown by persistence of the complex on electrophoresis (in contrast to the noncovalently linked complex), and resistance to exchange with corticosterone or pregnanediol and to solvent extraction. Site specificity of covalent binding was inferred from the effects of a scavenger, Tris·HCl, and fluorescence quenching of a neighboring tryptophan.**

Purification and definition of the properties of the corticosteroid receptors have been frustrated by their low abundance in cytoplasm and irreversible loss of binding activity during isolation (1–4). In principle, these problems could be circumvented by site-specific covalent labeling of the receptor (5, 6). In a previous study, 21-diazo derivatives of corticosteroids exhibited reasonable affinities for intracellular receptors and functional activity in a toad bladder assay (5). In addition, covalent labeling of plasma proteins was obtained with 9α-bromo-21-diazo-21-[*1,2*-^{3}H]deoxycorticosterone. The utility of this compound was limited, however, by the low specific activity (25 Ci/mol). We now report on the preparation of high-specific-activity 21-diazo-21-[*6,7*-^{3}H]deoxycorticosterone and site-specific, covalent labeling of human corticosteroid-binding globulin (CBG), used as a guide to future studies with steroid receptors.

MATERIALS AND METHODS

Precursor steroids were purchased from Upjohn; pregnanediol, cortisol, and corticosterone from Sigma; *d*-aldosterone from Calbiochem; and [*1,2*-^{3}H]corticosterone (54.5 Ci/mmol) from New England Nuclear. The conventional reagents were of the highest purity available (reagent grade or spectroquality). When necessary, the solvents used in steroid preparations were redistilled or purified by the method of Fieser (7). Purified human CBG was obtained as a generous gift from Dr. W. Rosner.

Preparation of 21-Diazo-21-[*6,7*-^{3}H]deoxycorticosterone. Thin-layer chromatography (TLC) was done as described (5) with the following solvent systems: system A, benzene-ethyl acetate (1:2 vol/vol); system B, benzene-ethanol (6:1 vol/vol); system C, chloroform-ethyl acetate (1:3 vol/vol). Melting points, microanalyses, and UV spectroscopy were performed as described (5). Catalytic tritiation was performed with the assistance of Dr. Chin-Tzu Peng and Mr. Ray Aune at the Lawrence Radiation Laboratory (Berkeley, Calif.). The reaction sequence used to prepare 21-diazo-21-[*6,7*-^{3}H]deoxycorticosterone (compound V) is shown in Fig. 1. Compound II was obtained by treatment of compound I (2.0 g) with chloranil (6.5 g) in *t*-butanol (120 ml). The filtered mixture was evaporated, taken up in ether, washed with dilute NaOH and water, and reevaporated. The residue was crystallized from aqueous methanol; mp 171–174° [lit. mp 155–177° (8)], λ_{max} 286 nm. Compound III was prepared by reaction of compound II (0.9 g in 10 ml of dioxane + 1.5 ml of H_2O) with sodium hypobromite (prepared from 0.3 ml of Br_2 and 0.5 g of NaOH in 8 ml of H_2O) at 0°. The reaction mixture was diluted with dioxane (4 ml), stirred for 2.5 hr at 0°, treated with $NaHSO_3$ (50 mg) for 15 min, extracted with ether, and acidified with HCl. The precipitate was washed with H_2O and crystallized from aqueous acetone (mp 270–275°, λ_{max} 286 nm) (Anal. calcd. for $C_{20}H_{26}O_4$: C, 72.70; H, 7.93. Found: C, 72.54; H, 7.83). Compound IV [mp 251–255°, λ_{max} 242 nm, (ϵ 1.55 × 10^4)] was prepared from compound I as described (5). Compound V [mp 175° (dec), λ_{max} 247 nm (ϵ 2.3 × 10^4)], with a shoulder at 280 nm, was prepared from compound IV as described (5). Tritium-labeled compound IV (at C_6–C_7) was obtained by catalytic tritiation of compound III in benzene-tetrahydrofuran solvent using 10% palladium on charcoal and carrier-free tritium gas in a microhydrogenation apparatus using vacuum line techniques. Analysis of the product by UV spectroscopy indicated the absence of compound III and a 45% yield of compound IV. A 2:3 mixture of the crude ^{3}H-labeled product and unlabeled compound IV was reacted with oxalyl chloride in benzene at 5°, and lyophilized. The residue was dissolved in benzene and treated with ethereal diazomethane under cooling by dry ice/acetone (9). After TLC twice in solvent systems A and B, purified tritiated compound V (21-diazo-21-[*6,7*-^{3}H]deoxycorticosterone) was recovered (36% yield) with a specific activity of 7 Ci/mmol and a purity of 98% (by TLC in system C). Identity was also confirmed by (*i*) photolysis at 253.7 and 300 nm, which gave the same results with ^{3}H product as with authentic compound V (Fig. 2) [note the rapid (<1 min) change in absorbance with compound V only]; (*ii*) the methyl esters (compound VI) formed on photolysis of labeled and unlabeled compound V in methanol gave identical R_F values on TLC; and (*iii*) when treated with HCl, both yielded compound VII, with R_F values on TLC (system A) identical to authentic compound VII prepared by the method of Reichstein and Fuchs (10).

Covalent Binding to CBG. Purified CBG was stored in aliquots at −20° (0.4 mg/ml, 0.05 M PO_4, pH 7.9) and contained

Abbreviations: CBG, corticosteroid binding globulin; TLC, thin-layer chromatography.

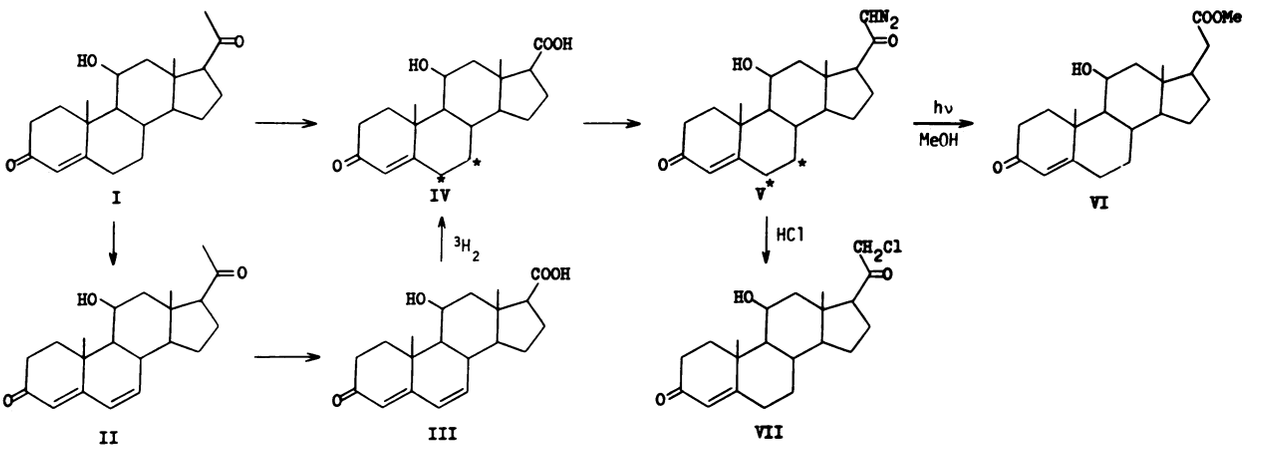

FIG. 1. Pathways in the synthesis of 21-diazo-21-[6,7-^{3}H]deoxycorticosterone. Compound I, 11β-hydroxypregn-4-ene-3,20-dione; compound II, 11β-hydroxypregn-4,6-diene-3,20-dione; compound III, 11β-hydroxy-3-oxo-androst-4,6-diene-17β-carboxylic acid; compound IV, 11β-hydroxy-3-oxo-androst-4-ene-17β-carboxylic acid; compound V, 21-diazo-11β-hydroxypregn-4-ene-3,20-dione (21-diazo-21-deoxycorticosterone); compound VI, methyl-11β-hydroxy-3-oxopregn-4-ene-21-oate; compound VII, 21-chloro-11β-hydroxypregn-4-ene-3,20-dione. Tritiation is denoted by asterisks (*).

[^{3}H]cortisol (2.88 μg/ml) to enhance stability (11). The contribution of [^{3}H]cortisol (48 dpm/μg) to radiolabeling of the CBG was negligible. Thawed aliquots were diluted 1:4 with glycerol-PO_4 buffer [0.13 M NaCl 0.05 M PO_4 (pH 7.9), and 40% (vol/vol) spectroquality glycerol] or the same solution containing 0.05 M Tris·HCl (pH 7.9) rather than PO_4 (glycerol-Tris buffer).

Five to 50 μl of the diluted solution of CBG (0.1 mg/ml) was diluted further to 1 ml (or to 0.2 ml if the product was to be analyzed by electrophoresis) with either the glycerol-PO_4 or the glycerol-Tris buffer (final concentrations of 0.5–5.0 μg of protein per ml) in a 1-cm light path quartz cuvette. All experiments were done either under red-light conditions or in a hood lined with opaque material to avoid exposure to direct light. Various concentrations of steroids (see legends) were added to the CBG, incubated for 15 min at 25° in a water bath, and then rapidly (in 2–3 min) reduced to −12° in a Neslab refrigerated bath. The super-cooled sample was transferred to a Rayonet photochemical reactor fitted with 16 (75 W) 253.7 nm UV lamps. A carousel rotated the samples during photolysis and dry ice set above the fan at the bottom of the chamber cooled the lamps during photolysis. The maximum temperature at the end of photolysis was −8°. The CO_2 vapors did not quench the photolytic reaction. The samples were then treated with 0.5 ml of 5% charcoal–0.5% dextran T180 (wt/vol) in either glycerol-PO_4 or glycerol-Tris buffer. The mixture was incubated at −12° for 10 min, and then centrifuged for 10 min at 27,000 × *g*. Parallel mixtures were also prepared without CBG to correct for [^{3}H]steroid that escaped adsorption to the charcoal.

Labeled CBG without prior treatment with charcoal was analyzed by polyacrylamide gel electrophoresis (7% total acrylamide, wt/vol) at 0° as described, except that sodium dodecyl sulfate and 2-mercaptoethanol were omitted (12).

Fluorescence Quenching. Five micrograms of CBG were diluted to 1 ml with glycerol-Tris buffer. Steroids at various concentrations (≦15 μl) were incubated with CBG, with or without photolysis as described above. The controls contained all constituents except CBG. Fluorescence quenching was assayed by excitation at 280 nm and emission at 350 nm (triplicate readings) in an Aminco-Bowman spectrophotofluorometer, at room temperature, as described by Stroupe *et al.* (13). Incubated samples were irradiated with UV light (−12°, 253.7 nm) and then warmed to room temperature for a repeat analysis of fluorescence quenching.

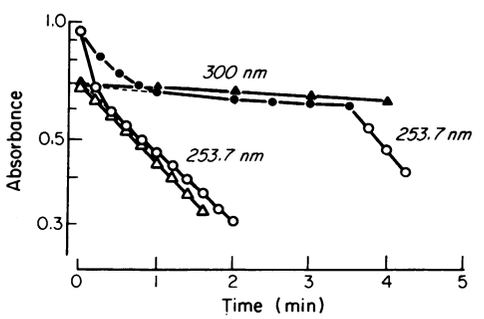

FIG. 2. Effect of UV irradiation on absorbance of 21-diazo-21-deoxycorticosterone (compound V) and 11β-hydroxypregn-4-ene-3,20-dione (compound I). The steroids in methanol (4.3 × 10^{-5} M) were irradiated in a quartz cell (1 cm light path) on a rotating stage of a Rayonet Photochemical Reactor at 25°, at 253.7 nm or 300.0 nm. Absorbance (max) was determined at 242–247 nm in a Beckman DB-G UV spectrophotometer. Absorbance of compound V after irradiation at 253.7 nm (○) and at 300.0 nm (●), and of compound I at 253.7 nm (△) and at 300.0 nm (▲).

RESULTS AND DISCUSSION

The photoaffinity technique requires the use of ligands with high affinity and selectivity for the primary binding site. To determine that 21-diazo-21-deoxycorticosterone had the requisite properties, we made analysis of the binding of this compound and of corticosterone (a natural ligand) to CBG. Preparations of CBG were incubated with steroids at 25° for 15 min at pH 7.9 and cleared with charcoal at −12°, because the rate of dissociation was minimal under these conditions. The affinity of the diazo derivative for CBG was determined by: (*i*) estimation of the apparent equilibrium dissociation constants (K_d) and maximum number of binding sites (N_{max}) for corticosterone and the diazo derivative by the method of Scatchard (14), and (*ii*) estimation of the half-maximal concentrations (K_I) for inhibition of binding of [^{3}H]corticosterone (5 × 10^{-8} M) to CBG using various concentrations of either unlabeled corticosterone or diazo derivative and computed from plots of % bound against logarithmic concentration of the competitor. As determined

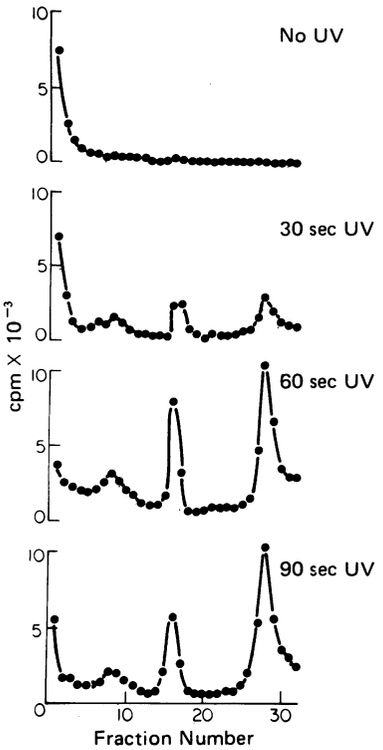

FIG. 3. Polyacrylamide gel electrophoresis of labeled CBG as a function of time of irradiation. A preparation of CBG (1 μg of protein per ml of glycerol-PO_4 buffer) was incubated with 21-diazo-21-[6,7-^{3}H]deoxycorticosterone for 15 min at 25°. The solutions were irradiated (253.7 nm) at −12° for 0–90 sec. The samples were analyzed by electrophoresis in 0.7 × 13 cm tubes, in Tris-glycine-glycerol buffer (pH 8.3) at 0°, as described (12). The sample was stacked with a current of 1 mA per gel for 15–20 min and then subjected to currents of 4 mA per gel for 3 hr. Slices (2 mm thick) were dissolved overnight in 0.4 ml of 30% H_2O_2 at 60°. Values represent ^{3}H (cpm/2 mm) in the separating gel, to the dye front. Migration is to the right.

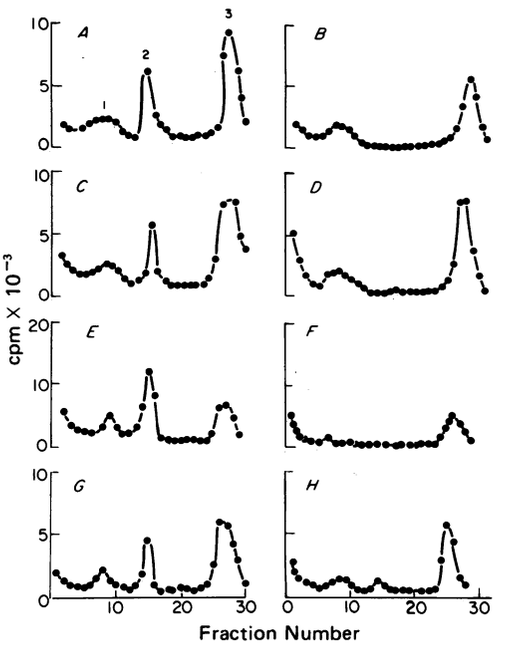

FIG. 4. Steroid specificity of labeling of CBG, before and after irradiation. A preparation of CBG (1 μg of protein per ml in glycerol-PO_4 buffer) was incubated for 15 min at 25° with either [^{3}H]corticosterone or 21-diazo-21-[6,7-^{3}H]deoxycorticosterone (2×10^{-7} M). Competitors (2×10^{-5} M) were added either before or after irradiation at −12° (90 sec, 253.7 nm). The products were analyzed by electrophoresis as described in the legend of Fig. 3. The steroid additions were as follows: (*A*) 21-diazo-21-[6,7-^{3}H]deoxycorticosterone alone; (*B*) 21-diazo-21-[6,7-^{3}H]deoxycorticosterone + corticosterone (before UV); (*C*) 21-diazo-21-[6,7-^{3}H]deoxycorticosterone + aldosterone (before UV); (*D*) 21-diazo-21-[6,7-^{3}H]deoxycorticosterone + cortisol (before UV); (*E*) [^{3}H]corticosterone alone; (*F*) [^{3}H]corticosterone + corticosterone (after UV); (*G*) 21-diazo-21-[6,7-^{3}H]deoxycorticosterone + corticosterone (after UV); (*H*) 21-diazo-21-[6,7-^{3}H]deoxycorticosterone + CBG that had been warmed to 60° for 5 min.

by these methods, the apparent K_d of corticosterone was 2.2×10^{-8} M and that of the 21-diazo-derivative was 3.2×10^{-8} M (ratio = 0.69). N_{max} was the same for both, 1.1×10^{-11} mol/μg of CBG. On the assumption of 90% purity (W. Rosner, personal communication) and a molecular weight of 51,700, this N_{max} is equivalent to 0.7 mol of steroid bound per mol of CBG, which approximates the molar ratio reported previously (15). The K_d values are designated "apparent" in that the presence of cortisol in the stock CBG solution may have biased the results. In previous studies, corticosterone had K_d values of 1.9×10^{-9} M (4°) and 1.60×10^{-7} M (37°), as compared to 2.2×10^{-8} M (25°) in the present study (15). K_I values for corticosterone and the 21-diazo derivative were 6.0×10^{-8} M and 8.2×10^{-8} M, respectively (ratio = 0.73). The crucial point, however, is that 21-diazo-21-deoxycorticosterone has the requisite high affinity and site specificity to qualify as a photoaffinity ligand for CBG.

To ensure that photodependent labeling was to CBG, the products were analyzed by gel electrophoresis. 21-Diazo-21-[6,7-^{3}H]deoxycorticosterone (2×10^{-7} M) was incubated with CBG at 25° and irradiated (−12°, 253.7 nm) for various intervals. Without irradiation no ^{3}H was detected in the gel, except for the diffusion front at the origin (Fig. 3). This is a consequence of dissociation from CBG during stacking. In contrast, three peaks (R_F 0.25, 0.5, and 0.85 with respect to the dye front) were apparent after 30, 60, and 90 sec of irradiation, with a maximal yield after 60 sec. Under these electrophoretic conditions, CBG has an R_F of about 0.5 (16).

To test the specificity of labeling, we incubated the CBG preparation with 2×10^{-7} M 21-diazo-21-[6,7-^{3}H]deoxycorticosterone with or without 100-fold excess of unlabeled corticosterone, cortisol, or aldosterone prior to UV irradiation. The results confirm the presence of three peaks of ^{3}H activity (Fig. 4A). Only the middle peak was eliminated by glucocorticoids that bind to CBG: corticosterone (Fig. 4B) and cortisol (Fig. 4D) (15). In contrast, aldosterone (Fig. 4C), a steroid with very little affinity for CBG, had no effect on the labeling pattern (15). These results show that the binding of the ^{3}H-labeled diazo compound to CBG had the requisite specificity. Peaks and 3 (R_F 0.25 and 0.85, respectively) were unaffected by the competing steroids.

The stability of photoaffinity labeling of CBG was tested in a further series of experiments. The CBG preparation was incubated with [^{3}H]corticosterone or 21-diazo-21-[6,7-^{3}H]deoxycorticosterone (2×10^{-7} M) and then irradiated. The irradiated preparations were re-incubated with 100-fold excess of unlabeled corticosterone for 15 min at 25° and the products were analyzed by gel electrophoresis. Both [^{3}H]steroids labeled the material of peak 2 prominently, as well as that of peaks 1 and 3 (Fig. 4A and 4E). Addition of corticosterone after irradiation eliminated binding of [^{3}H]corticosterone to peak 2 material (Fig. 4F), but had a minimal effect on labeling with

Table 1. Effect of duration of UV irradiation on exchange of [^{3}H]corticosterone and 21-diazo-21-[6,7-^{3}H]-deoxycorticosterone bound to CBG

[^{3}H]Steroid (M)	Corticosterone (M) after UV	[^{3}H]Steroid bound to CBG* 0 sec	30 sec	60 sec	90 sec
[^{3}H]Cortico-sterone	0	4.00	3.21	2.83	1.73
	10^{-5}	−0.21	−0.18	0.10	0.03
	% Exchange	~100	~100	97	98
^{3}H-labeled diazo derivative	0	3.30	2.11	1.42	1.16
	10^{-5}	0.02	0.93	1.23	1.71
	% Exchange	100	56	13	<0

The [^{3}H]steroid (10^{-7} M) was incubated with 0.5 μg of CBG in glycerol-PO_4 buffer for 15 min at 25° and irradiated at −12° (253.7 nm) for 0–90 sec. Unlabeled corticosterone or the diluent was added to the solutions and the cuvettes were re-incubated for 15 min at 25°. Bound steroid was assayed by the charcoal-dextran method.

* Picomoles of [^{3}H]steroid bound per μg of protein. n = 5. A negative value indicates less ^{3}H activity than in the CBG-free controls.

the diazo [^{3}H]steroid (Fig. 4G), which indicates that photodependent labeling with the ^{3}H-labeled diazo derivative is either covalent or dissociates at a very slow rate. Binding of the ^{3}H-labeled diazo derivative required the native state, in that heating of the CBG preparation to 60° for 5 min before incubation and irradiation eliminated labeling of CBG but not of material in peaks 1 and 3 (Fig. 4H). This thermostability experiment was also prompted by the possibility that peak 2 contained albumin rather than CBG because albumin is a scavenger in photolytic reactions and also has an R_f of about 0.5 under these conditions (17). Inasmuch as CBG is inactivated at 60° and albumin is not, the loss of binding in Fig. 4H confirms that peak 2 contains CBG.

The stability of the photoproduct was explored with studies on exchange-resistance. CBG preparations were incubated in [^{3}H]corticosterone or 21-diazo-21-[6,7-^{3}H]deoxycorticosterone (10^{-7} M) in glycerol-PO_4 buffer and irradiated. The samples were then reincubated at 25° for 15 min, with or without a 100-fold excess of unlabeled corticosterone. Before and after irradiation, all of the bound [^{3}H]corticosterone was exchangeable (Table 1), whereas the bound ^{3}H-labeled diazo derivative was progressively less exchangeable in proportion to the duration of irradiation.

Further evidence that the ^{3}H-labeled diazo derivative is covalently bound to CBG was obtained in solvent extraction studies. Duplicate samples of glycerol-Tris buffer with or without CBG were incubated with 2×10^{-7} M [^{3}H]corticosterone or 21-diazo-21-[6,7-^{3}H]deoxycorticosterone and irradiated. Free steroid was removed with charcoal-dextran, leaving 2.8×10^{-12} mol bound per μg of protein. Aliquots were then extracted with either methylene dichloride (once) or ethyl acetate (twice) as described (18). The solvent: H_2O partition coefficients computed from extractions of protein-free solutions were 0.18 for both steroids in CH_2Cl_2, and 0.24 for corticosterone and 0.36 for the diazo derivative in $CH_3COOC_2H_5$. Methylene dichloride extracted 83% of the [^{3}H]corticosterone and only 6% of the ^{3}H-labeled diazo derivative. Similarly, ethyl acetate extracted 90% (85 + 5) of the [^{3}H]corticosterone and only 21% (20 + 1) of the ^{3}H-labeled diazo derivative. These results imply covalent binding of the diazo derivative to CBG.

To explore the issue of site-specific labeling, we exploited two techniques: (*i*) the effects of a scavenger on photoaffinity labeling of the CBG, and (*ii*) fluorescence quenching analysis. Photolysis of the diazoketones produces the corresponding ketene capable of acylating amines (19). Accordingly, Tris·HCl was substituted for the PO_4 in the buffer to provide an excess of amines as reactants for photoactivated ^{3}H-labeled diazo derivative and thereby minimize secondary labeling of the CBG. Aliquots of the CBG preparation were incubated with 21-diazo-21-[6,7-^{3}H]deoxycorticosterone (2×10^{-7} M) in glycerol-PO_4 or glycerol-Tris buffer. The preparations were irradiated and the products analyzed by electrophoresis. Photolysis in PO_4 buffer produced the usual labeling pattern (Fig. 5A). In the presence of Tris, however, the major nonspecific component, in peak 3, was essentially eliminated, whereas labeling of peak 2 material (CBG) was augmented to some extent (Fig. 5B). These results imply that covalent attachment ensued in the bound state rather than from derivatives with access to the solvent.

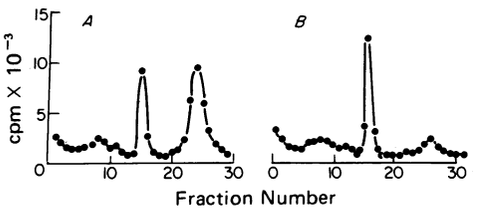

FIG. 5. Effect of a scavenger, Tris·HCl, on photoaffinity labeling of CBG. A preparation of CBG (1 μg of protein per ml) was incubated with 21-diazo-21-[6,7-^{3}H]deoxycorticosterone (2×10^{-7} M) in either (A) glycerol-PO_4 buffer or (B) glycerol-Tris buffer for 15 min at 25°. All incubated samples were irradiated at −12° (90 sec, 253.7 nm) and then analyzed by electrophoresis as described in the legend of Fig. 3.

A test for assessing site-specific steroid occupancy is the fluorescence quenching technique (13). Steroid binding in close proximity to a fluorescence donor (e.g., tryptophan in CBG) results in quenching if the absorption spectrum of the steroid acceptor overlaps extensively with the emission spectrum of the donor. The efficiency of fluorescence quenching depends on the distance between donor and acceptor by the relationship:

$$E = R_0^6/(R^6 + R_0^6) \quad [1]$$

where E denotes the efficiency of quenching, R_0 the distance in Å for equal probability of radiationless energy transfer, and R the distance in Å between donor and acceptor. In previous studies with progesterone-binding globulin, both deoxycorticosterone and progesterone, which contain Δ^4-3 keto functions (sites of energy absorption), were efficient quenchers (13). Because the CBG preparation used in the current studies contained cortisol, it was necessary to replace this steroid with one that would bind to the site but had minimal quenching activity. Stroupe *et al.* (13) introduced the use of 5β-pregnane-3,20-dione (pregnanedione) as a "see-through" steroid since it lacks the Δ^4-3 keto structure. For these studies we have substituted pregnanediol. The first set of experiments consisted of three steps. (*i*) Pregnanediol (10^{-4} M) was added to CBG (5 μg of protein per ml) or the diluent in glycerol-Tris buffer. (*ii*) Corticosterone or the 21-diazo derivative or the diluents were added in successive increments to pairs of cuvettes. Triplicate readings were begun 5 min after each addition. Fluorescence quenching reached the same limiting value (56% of control) at 10^{-6} to 4×10^{-6} M of either steroid, implying that both occupied the same site (Fig. 6A). (*iii*) Samples in all cuvettes were then irradiated and reassayed. Irradiation had no effect on fluorescence quenching with either steroid, indicating unchanged orientations of the CBG fluorophore and steroid chromophores.

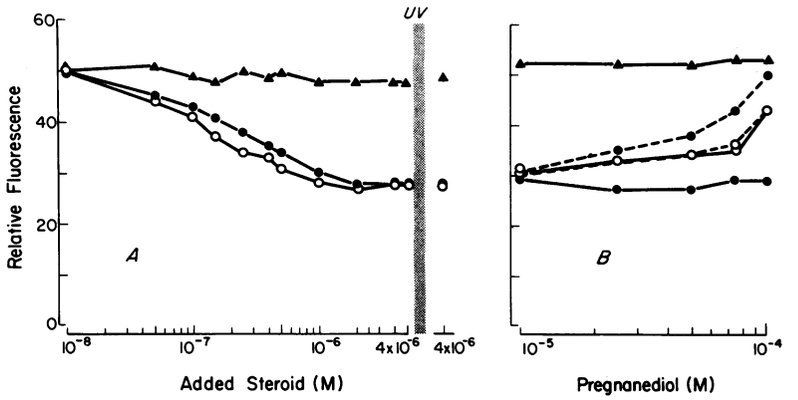

FIG. 6. Fluorescence quenching of CBG by corticosterone and 21-diazo-21-deoxycorticosterone. (A) Tandem quartz cuvettes contained 5 μg/ml of CBG or the diluent and 10^{-4} M pregnanediol in Tris-glycerol buffer. Serial additions of corticosterone (○), 21-diazo-21-deoxycorticosterone (●), or the diluent (▲) were made to pairs of cuvettes. Fluorescence was monitored after each addition by activation at 280 nm and emission at 350 nm (room temperature). After the last addition (4×10^{-6} M) samples in all cuvettes were irradiated at −12° (60 sec, 253.7 nm) and reassayed for fluorescence at room temperature. (B) Cuvettes contained 5 μg/ml of CBG or the diluent in Tris-glycerol buffer. Pregnanediol (10^{-4} M) was added to one pair of cuvettes and monitored with each assay to control for time-dependent variations in the fluorescence yield (▲). Corticosterone (○) and 21-diazo-21-deoxycorticosterone (2×10^{-7} M) (●) were added to pairs of cuvettes and incubated for 15 min at 25°. One of each pair of cuvettes (solid lines) was irradiated at −12° as above and assayed for fluorescence quenching. The other pairs were not irradiated (dashed lines). Cuvettes that contained corticosterone or the 21-diazo derivative were then supplemented with stepwise additions of pregnanediol (up to 10^{-4} M) and assayed for fluorescence yield between each addition. Relative fluorescence (ordinate) denotes the difference in the fluorescence yields of identical solutions with and without CBG.

To ensure that photolysis resulted in irreversible binding of the 21-diazo derivative to the primary site, the reversal of quenching by exchange with pregnanediol was assessed. CBG preparations (5 μg of protein per ml) or the diluent in glycerol-Tris buffer were incubated in pairs with corticosterone (2×10^{-7} M) or 21-diazo derivative (2×10^{-7} M) or pregnanediol (10^{-4} M), for 15 min at 25°. One of each pair of cuvettes was irradiated and the fluorescence yield determined. Corticosterone and the 21-diazo derivative quenched fluorescence to the same extent with and without irradiation (points at the origin) (Fig. 6B). Additions of pregnanediol reversed corticosterone quenching regardless of irradiation and that of the nonirradiated 21-diazo derivative. No reversal of quenching was obtained in the irradiated complexes of 21-diazo derivative (Fig. 6B).

The results in Fig. 6 enable an estimation of R, the distance between donor and acceptor (Eq. 1). To do so, R_0 was computed from Forster's equation (20)

$$R_0 = 9.79 \times 10^3 (K^2 n^{-4} Q J)^{1/6} \quad [2]$$

The following values were used: K^2 (dipole–dipole orientation factor) = 4 (13); n (refractive index of medium at site) = 1.5 (13); Q [fluorescent quantum yield of donor determined relative to tryptophan standard (0.13)] = 0.119; and J (integral of the spectral overlap of the acceptor determined in methanol, and the donor) = 2.5×10^{-19} cm^6 mmol^{-1}. These values yield R_0 = 5.2 Å and R (Eq. 1) = 5.4 Å. Noncovalently bound diazo derivative and corticosterone, as well as the covalently bound steroid, had the same spatial relationships to the donor, R = 5.4 Å (Fig. 6).

In summary, photoactivation of 21-diazo derivative:CBG results in covalent attachment to the primary binding site. This technique hopefully should contribute to purification and characterization of intracellular glucocorticoid receptors as well.

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