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Retinoid Affinity Label for the Binding Site of Retinol-Binding Protein†

Mary Ann Gawinowicz and DeWitt S. Goodman*

ABSTRACT: Three radioactive retinoid bromoacetates were synthesized as potential retinoid affinity labels for the retinol binding site of human plasma retinol-binding protein (RBP). The compounds synthesized were β-[9-^{3}H]ionyl bromoacetate (IBA), β-[11-^{3}H]ionylideneethyl bromoacetate (IEBA), and [15-^{3}H]retinyl bromoacetate (RBA). When excess ligand was incubated with RBP for 5 h at 37 °C, IBA and IEBA formed nearly 1:1 molar complexes with RBP, whereas RBA bound only approximately one-third as well. Subsequent addition of retinol to the retinoid–RBP complex resulted in complete displacement of IBA and RBA from the protein, whereas a large proportion (37%) of the [^{3}H]IEBA remained bound to the retinol binding site of RBP. For maximization of covalent bonding of IEBA to RBP, IEBA was incubated with RBP for varying lengths of time, followed, in each instance, by addition of retinol to displace noncovalently bound IEBA. The amount of IEBA remaining bound to RBP increased with increasing incubation time, reaching a maximum of about 0.66 mol/mol of RBP at 18 h. Moreover, at each time point, the binding of retinol to RBP was inhibited to an extent that was equivalent to the amount of [^{3}H]IEBA that was not displaced from RBP by retinol. Only a very small proportion of the bound [^{3}H]-IEBA that was not displaced with retinol could be extracted from the protein with chloroform–methanol. Taken together, these several lines of evidence strongly suggest that the IEBA was bound in the retinol binding site of RBP and was attached to the protein in a covalent manner. Thus, IEBA appears to be an effective affinity label for the retinol binding site of RBP.

Retinol (vitamin A) circulates in plasma bound to a specific transport protein, retinol-binding protein (RBP) (Kanai et al., 1968). RBP is a single polypeptide chain with a molecular weight of approximately 20 000 [see Goodman (1980) and Smith & Goodman (1979) for recent reviews]. The primary structure of human plasma RBP is now known (Rask et al., 1979, 1981; Kanda & Goodman, 1979). RBP serves to mobilize vitamin A from its stores in the liver and to deliver it to peripheral target tissues. RBP has a single binding site for one molecule of retinol and circulates mainly as the retinol–RBP complex. RBP also interacts strongly with plasma prealbumin and normally circulates as a 1:1 molar RBP–prealbumin complex.

The binding of *all-trans*-retinol to RBP is reversible and fairly specific. A variety of retinoids can bind to apo-RBP with varying degrees of effectiveness [see Goodman (1980) for review and references]. Some of these (e.g., *all-trans*-retinoic acid) (Cogan et al., 1976) bind to RBP with an affinity similar to that of retinol. Other retinyl derivatives have also been shown to bind well to RBP, including compounds with shortened side chains such as β-ionone and β-ionylideneacetic acid (Hase et al., 1976). It appears from these studies that some degree of structural similarity to retinol, particularly in the area of the cyclohexene ring, is necessary for binding to RBP.

These studies with retinoids have provided some information about the structural requirements of the retinol binding site on RBP. No information is, however, available about the amino acid residues in RBP that are involved in the binding site. Acetylation of lysine residues of RBP did not affect its binding of retinol (Heller & Horwitz, 1975). Modification of one of eight tyrosine residues and two of four tryptophan residues of RBP also had no effect on the retinol–RBP interaction (Heller & Horwitz, 1975; Horwitz & Heller, 1974b). The binding site was, however, disrupted by reduction and alkylation of disulfide bonds (Raz et al., 1970).

This paper reports the results of the first phase of a retinoid affinity labeling study of the retinol binding site of RBP. The goal of the work reported here was to synthesize a retinoid with a reactive functional group that would bind reversibly to the retinol binding site of RBP and would then react and form a covalent bond with proximate amino acid residues. The reactive retinoid would thus serve as a label of the binding site. Subsequently, proteolytic cleavage and peptide mapping of the labeled RBP should yield one or more peptides containing the label, thus identifying them as having some involvement in the retinol binding site.

We now report the synthesis of three retinoid bromoacetates that comprise a homologous series of compounds with varying chain lengths. We also report studies of the binding of these three retinoids to RBP, together with data which demonstrate the covalent binding of one of the compounds to the retinol binding site of RBP.

Materials and Methods

Synthesis of Retinoid Bromoacetates. Radioactive compounds that were structurally similar to retinol and contained the bromoacetyl functional group were prepared and tested for their ability to bind to RBP. The three compounds synthesized and studied were retinyl bromoacetate (Figure 1, RBA), the bromoacetyl analogue of retinol, and two compounds with shorter side chains, β-ionyl bromoacetate (Figure 1, IBA), and β-ionylideneethyl bromoacetate (Figure 1, IEBA). All three analogues were prepared from the respective

† From the Department of Medicine, College of Physicians and Surgeons of Columbia University, New York, New York 10032. *Received October 8, 1981.* This research was supported by Grants HL 21006 (SCOR in arteriosclerosis) and AM 05968 and by Training Grant HL 07343 (M.A.G.) from the National Institutes of Health.

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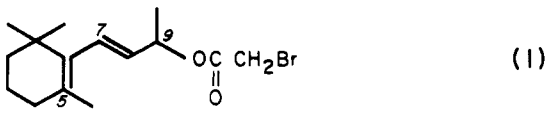
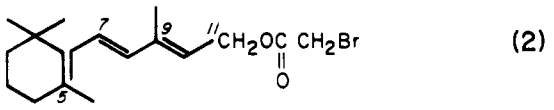


(1)

β-Ionylbromoacetate (IBA)



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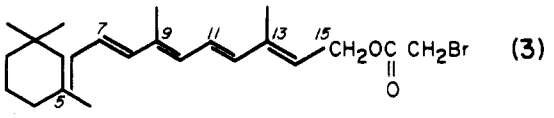
β-Ionylideneethanol bromoacetate (IEBA)



(3)

Retinyl bromoacetate (RBA)

FIGURE 1: Retinoid bromoacetates prepared for use as potential affinity labels for RBP. Compounds were labeled with tritium at C-9 (IBA), C-11 (IEBA), and C-15 (RBA).

nonradioactive aldehyde or ketone precursor which was reduced to the alcohol with sodium borotritide and then bromoacetylated.

β-[9-^{3}H]Ionyl Bromoacetate. To a slurry of sodium borotritide (New England Nuclear; 246 mCi/mmol, 15 mg, 0.40 mmol) in 10 mL of 2-propanol was added a solution of β-ionone (0.40 g, 2.1 mmol) in 10 mL of 2-propanol. After the mixture was stirred for 2 h at room temperature, sodium borohydride (110 mg, 3.0 mmol) was added to reduce unreacted β-ionone. Stirring was continued for 18 h, and water was added. The layers were separated, and the organic phase was washed with a saturated NaCl solution and water and dried over $MgSO_4$. Filtration through silica gel (ether–hexane, 1:4 v/v) gave 0.32 g (1.6 mmol) of β-[9-^{3}H]ionol.

The bromoacetate was prepared by dissolving β-[9-^{3}H]ionol in 20 mL of benzene and bringing the solution to 0 °C in an ice bath. To this was added 0.5 mL of pyridine, followed by 0.25 mL of bromoacetyl bromide. The reaction mixture was allowed to stir for 30 min at 0 °C and then was diluted with 10 mL of ether. The solution was washed successively with water, dilute H_2SO_4, and saturated $NaHCO_3$ solution and was dried over $MgSO_4$. The product, β-[9-^{3}H]ionyl bromoacetate (0.24 g, 0.76 mmol, 29 mCi/mmol), was isolated after chromatography on alumina (ether–hexane, 1:9 v/v). The absorption spectrum (ethanol) showed a peak at 234 nm (ϵ = 1680). The NMR spectrum ($CDCl_3$) showed signals at δ 5.30 (t, CHO), 3.74 (s, CH_2Br), 1.59 (C-9 methyl), 1.32 (s, C-5 methyl), and 0.91 (*gem*-dimethyl).

β-[11-^{3}H]Ionylideneethyl Bromoacetate. Ethyl β-ionylideneacetate was synthesized from β-ionone via a Horner reaction with ethyl (diethylphosphono)acetate (Ishikawa, 1963). To a stirred suspension of sodium amide (0.60 g, 15.6 mmol) in 30 mL of dry tetrahydrofuran under nitrogen was added ethyl (diethylphosphono)acetate (4.2 g, 18.7 mmol) in 10 mL of tetrahydrofuran. The mixture was stirred at room temperature for 30 min and then β-ionone (3.0 g, 15.6 mmol) was added in 10 mL of tetrahydrofuran over a 30-min period. After an additional 1 h at room temperature, the reaction mixture was heated at 40 °C overnight. The flask was cooled, and the solution was diluted with diethyl ether and then washed with water and saturated NaCl. The aqueous layer was extracted twice with ether, and the ether extracts were dried over $MgSO_4$. Filtration and concentration were followed by silica gel chromatography (ether–hexane, 1:9 v/v) which yielded pure ethyl β-ionylideneacetate as a colorless oil (2.6 g, 9.9 mmol). The absorption spectrum (ethanol) contained two peaks at 300 (ϵ = 14900) and 255 nm (ϵ = 12400). The NMR spectrum ($CDCl_3$) showed signals at δ 4.15 (q, ethyl $-CH_2$), 2.30 (s, C-9 methyl), 1.66 (s, C-5 methyl), 1.25 (t, ethyl CH_3), and 1.03 (s, *gem*-dimethyl).

To a slurry of $LiAlH_4$ (0.52 g, 13.8 mmol) in 50 mL of anhydrous ether was added ethyl β-ionylideneacetate (2.4 g, 9.2 mmol) in 15 mL of diethyl ether. The reaction was stirred under nitrogen for 1 h and then cooled in an ice bath. A saturated solution of tartaric acid was carefully added dropwise until no further reaction occurred. At this time, 100 mL of water was added, and the layers were separated. The aqueous layer was extracted with ether, and the combined ether extracts were washed with water and dried over $MgSO_4$.

The concentrated alcohol was converted to the aldehyde by dissolving in 50 mL of CH_2Cl_2 and adding 20 g of freshly prepared activated MnO_2 (Attenburrow et al., 1952). The mixture was kept in dim light and stirred overnight at room temperature. The solution was filtered and concentrated under reduced pressure. Chromatography of 0.4 g (1.8 mmol) of crude material on silica gel (ether–hexane, 1:19 v/v) afforded 0.15 g (0.69 mmol) of β-ionylideneacetaldehyde. The aldehyde was reduced with sodium borotritide (116 mCi/mmol, 32 mg, 0.84 mmol) as described above for β-[9-^{3}H]ionol to yield, after chromatography on silica gel (ether–hexane, 1:5 v/v), 0.12 g (0.54 mmol) of β-[11-^{3}H]ionylideneethanol.

The alcohol was converted to β-[11-^{3}H]ionylideneethyl bromoacetate (38 mCi/mmol) as described above for β-[9-^{3}H]ionyl bromoacetate. Due to the extreme instability of the compound on silica gel and alumina, the reaction product was not further purified. It migrated, however, as a single spot on a thin-layer silica gel plate; this spot contained 85% of the total radioactivity. The absorption spectrum (ethanol) showed two peaks at 266 (ϵ = 12000) and 235 nm (ϵ = 12100). The NMR spectrum ($CDCl_3$) showed a pure compound with signals at δ 4.80 (d, CH_2O), 3.82 (s, CH_2Br), 1.86 (s, C-9 methyl), 1.65 (s, C-5 methyl), and 0.99 (s, *gem*-dimethyl).

[15-^{3}H]Retinyl Bromoacetate. all-trans-Retinol (Sigma; 0.76 g, 2.6 mmol) was converted to retinaldehyde with 7.6 g of MnO_2 as described above for β-ionylideneacetaldehyde. After chromatography on silica gel (ether–hexane, 1:9 v/v), the resulting *all-trans*-retinaldehyde was crystallized from petroleum ether.

Retinaldehyde (0.06 g, 0.21 mmol) was reduced with sodium borotritide (250 mCi/mmol, 15 mg, 0.4 mmol) as described above for β-[9-^{3}H]ionol. Column chromatography on alumina using 1:4 (v/v) ether–hexane afforded [15-^{3}H]retinol (80 mCi/mmol).

[15-^{3}H]Retinol (10 mg, 0.03 mmol) was converted to its bromoacetate derivative with bromoacetyl bromide by the method used to prepare β-[9-^{3}H]ionyl bromoacetate described above. Chromatography on silica gel using 1:19 (v/v) ether–hexane yielded [15-^{3}H]retinyl bromoacetate (80 mCi/mmol). The absorbance spectrum (ethanol) showed a peak at 325 nm (ϵ = 47200), and the NMR spectrum ($CDCl_3$) showed signals at δ 4.82 (d, CH_2O), 3.83 (s, CH_2Br), 1.97 (s, C-13 methyl), 1.90 (s, C-9 methyl), 1.70 (s, C-5 methyl), and 1.05 (s, *gem*-dimethyl).

RBP–Retinoid Binding Studies. Binding studies were carried out in two stages. In the first stage, a large excess of the radioactive retinoid was added to apo-RBP, in order to ensure maximal binding of the ligand to the protein. After

removal of excess ligand by gel filtration, the amount of retinoid bound was estimated by radioactivity (±spectrophotometric) assay. In the second stage, experiments were conducted to demonstrate that the bound retinoid was present in the retinol binding site and to determine whether or not covalent bonding had occurred. These experiments first involved addition of retinol to the RBP–ligand complex to displace the radioactive retinoid; the extent of displacement was then determined. Covalent binding of the radioactive retinoid to the retinol binding site of RBP was determined by (i) the amount of radioactive ligand which was not displaced by retinol, (ii) the degree to which retinol binding was inhibited, and (iii) the resistance of the radioactive retinoid to extraction from the protein.

Binding of Radioactive Analogues to RBP. Apo-RBP (0.8–1.3 mg) was dissolved in 1 mL of 0.02 M phosphate buffer, pH 7.5. To this solution was added, via a microsyringe and with stirring, a 100-fold molar excess of ligand [(4–6) × 10^{-6} mol] in ethanol. The volume of ethanol added was 2–5% of the total volume of the solution; the use of less ethanol led to inadequate dispersion of the ligand in the aqueous solution. The solution was gently shaken at 37 °C for 5 h and was then applied to a Sephadex G-100 column (1.5 × 45 cm). Elution with 0.02 M phosphate buffer, pH 7.5, resulted in the separation of the protein (RBP) from excess ligand. The eluate was monitored for absorbance (at 280 nm) and for radioactivity (^{3}H), and fractions containing RBP were combined and used for further study.

Binding of Retinol to Apo-RBP and the Retinoid–RBP Complex. The protein concentration of the combined fractions containing the retinoid–RBP complex was estimated from the absorbance at 280 nm. A control solution of apo-RBP was prepared so that its protein concentration was the same as that of the retinoid–RBP solution [(6.7–8.9) × 10^{-6} M]. Retinol was added to both protein solutions under the same conditions, so as to provide a direct comparison of the extent of retinol binding to the retinoid–RBP complex as compared to apo-RBP alone. To 1 mL of protein solution, a 3–4-fold molar excess of retinol was added in 10–20 μL of ethanol with stirring. The solution was shaken gently at 37 °C for 1 h and then gel filtered on a column of Sephadex G-25 (1.0 × 20 cm) to remove the excess retinol. Absorbance of the eluted fractions was determined at 280 nm and at 325 nm. Protein concentration was estimated from 280-nm absorbance, and the extent of retinol binding was determined by the absorbance ratio (A_{325}/A_{280}). In the case of RBP containing a bound affinity label, the protein fractions were assayed for radioactivity and then subjected to total lipid extraction.

Extraction of Noncovalently Bound Affinity Label. The method of Folch et al. (1957) was used to extract noncovalently bound ligand from the RBP solution. A 0.25-mL sample of the RBP solution was extracted with 20 volumes (5 mL) of a 2:1 (v/v) chloroform–methanol mixture. After addition of 1 mL of water, the two phases were separated by centrifugation, the chloroform phase was removed, and the aqueous phase was extracted again with 3.6 mL of chloroform–methanol–water (86:14:1 v/v/v). The chloroform extracts were combined and evaporated with N_2, and the resulting residue was assayed for radioactivity.

Correction for Ligand Absorbance. It was necessary to make a correction for ligand absorbance at 280 nm in calculating protein concentration for the RBP–IEBA complex. This was done by determining the amount of radioactive compound remaining with the protein after lipid extraction, which was taken to represent covalently bound IEBA in the retinol binding site of RBP. From this, the concentration of IEBA in the RBP binding site could be calculated, and when an ϵ of 10500 was used, the contribution of the ligand to the absorbance at 280 nm could be determined. The corrected protein absorbance and concentration values were then used to determine the molar ratios of retinol and of the affinity label to RBP.

Other Methods and Materials. The human RBP used in this study was the same preparation of RBP which had been used in the studies on the amino acid sequence of RBP previously reported from this laboratory (Kanda & Goodman, 1979). The isolated, lyophilized RBP had been stored at −20 °C. Protein concentrations were determined from absorbance at 280 nm by using a value of $E^{1\%}_{1cm}$ = 19.4 for RBP (Kanai et al., 1968).

Retinol used in the RBP binding studies was a generous gift from Drs. W. E. Scott and B. Pawson of Hoffmann-La Roche, Nutley, NJ.

Absorbance and absorption spectra were obtained by using a Beckman DB spectrophotometer. Radioactivity (^{3}H) levels were assayed in a Packard Model 3255 liquid scintillation counter. NMR spectra were recorded in a Bruker (WM 300) spectrometer.

Results

Preparation of Radioactive Bromoacetate Retinoids. The synthesis of the bromoacetate derivatives of retinol, and of two shorter analogues of retinol, was carried out in a straightforward manner by treating the appropriate alcohol with bromoacetyl bromide. In each case, the bromoacetate was the sole reaction product and was stable in solution in ethanol; however, in the cases of RBA and IEBA, there occurred marked decomposition when the compound was exposed without solvent to air or was in prolonged contact with silica gel or alumina.

As indicated below, one of these three compounds (β-ionylideneethyl bromoacetate, IEBA) proved to be an effective affinity label for the retinol binding site of RBP. This compound (IEBA) was prepared from ethyl β-ionylideneacetate which was synthesized from β-ionone. A Horner reaction with ethyl (diethylphosphono)acetate in the presence of sodium amide in tetrahydrofuran yielded the trans isomer of ethyl β-ionylideneacetate as confirmed by the proton NMR spectrum. The 7,8-protons give rise to an AB quartet with peaks at δ 6.50, 6.55 (J = 15 Hz) and 6.08, 6.03 (J = 15 Hz) consistent with the trans configuration around the 9,10-double bond (Mousseron-Canet et al., 1966). Conversion of the aldehyde was accomplished by treatment with $LiAlH_4$ (to yield the alcohol), followed by oxidation (to the aldehyde) with MnO_2. Reduction to the tritiated alcohol was then achieved with sodium borotritide.

Binding of Radioactive Retinoids to RBP. The binding of each of the three bromoacetate derivatives to apo-RBP was explored by addition of a 100-fold molar excess of the compound to a solution of RBP (see Materials and Methods). After incubation for 5 h at 37 °C, the excess ligand was removed by gel filtration through Sephadex G-100, and the ratio of bound ligand to protein was determined by measuring the level of ^{3}H associated with the protein.

The amount of *all-trans*-retinol bound to RBP can be determined from the ratio of absorbance at 325 nm (due to retinol bound to RBP) to that at 280 nm (due to the protein itself). This ratio is 1.05 for an equimolar complex of retinol and RBP (Kanai et al., 1968). It has been demonstrated that retinol specifically bound to RBP retains virtually the same absorption spectral properties as does retinol in solution in

Table I: Binding of Retinoid Bromoacetate to RBP and Subsequent Displacement by Retinol

ligand	^{3}H-labeled ligand bound to RBP[a] (mol/mol)	ligand bound to RBP after retinol addition[b] (mol/mol)	
		^{3}H-labeled ligand	retinol
IBA	1.04	0.08	0.90
IEBA	0.89	0.37	0.59
RBA	0.33	0.06	0.92

[a] Before addition of retinol. [b] Addition of retinol to apo-RBP (rather than to a retinoid–RBP complex) yielded a retinol–RBP complex with a mole ratio of 0.93 ± 0.03 (mean ± SD).

organic solvents and that the bound retinol is responsible for the peak of absorbance at 325–330 nm (Kanai et al., 1968; Goodman & Raz, 1972).

Retinyl bromoacetate has the same absorption maximum as retinol, so that the molar ratio of RBA bound to RBP could be calculated from the absorption spectrum of the complex as well as from the amount of radioactivity (^{3}H) bound to the protein. The absorbance maxima of the retinoids with shorter side chains were, however, obscured by the protein peak of absorbance. For IBA and IEBA, therefore, the extent of binding to RBP was determined solely from the amount of bound ^{3}H associated with the protein. As shown in Table I, first column, both shorter compounds formed nearly equimolar complexes with RBP, whereas RBA bound only one-third as well as did retinol.

Displacement of Bound Radioactive Retinoids by Retinol. Experiments were carried out to determine the extent to which the radioactive retinoid bound to RBP could be displaced by retinol. The results are shown in columns 2 and 3 of Table I. For both RBA and IBA, the retinoid ligand was almost completely displaced by retinol. Thus, after incubation of the retinoid–RBP complex with added retinol, only 0.06 and 0.08 mol of radioactive retinoid, respectively, for the two compounds, remained bound to RBP. In these experiments the added retinol was found to bind fully to the RBP, with mole ratios (retinol:RBP) of 0.92 and 0.90, respectively. These latter values were similar to those observed for the binding of retinol added directly to apo-RBP under identical conditions (mole ratio 0.93 ± 0.03, mean ± SD for 16 such determinations). These results suggested that both radioactive retinoids (RBA and IBA) had been bound in the retinol binding site but indicated that covalent bonding had not occurred (since the labeled ligands could be displaced by retinol, with no inhibition of formation of the retinol–RBP complex).

Quite different results were obtained with IEBA. Thus, after addition of retinol to the IEBA–RBP complex, 0.37 mol of radioactive retinoid remained bound to the protein (Table I, column 2), and the binding of retinol to the RBP was correspondingly reduced (Figure 2 and Table I, column 3). These data indicated that the radioactive retinoid in the binding site of RBP inhibited the binding of retinol and suggested that covalent bonding of the labeled retinoid to RBP had occurred.

The effect of the time of incubation of IEBA with RBP on the subsequently observed inhibition of the binding of retinol to the RBP is shown in Table II. Binding of [^{3}H]IEBA to RBP was carried out with incubation times ranging from 2.5 to 24 h. Although a complex comprised of 0.89 mol of IEBA/mol of RBP was formed by 5 h (Tables I and II), it was thought that increased incubation time might allow the bromoacetyl group of the ligand within the binding site to react with a nearby amino acid residue to a greater extent, thus

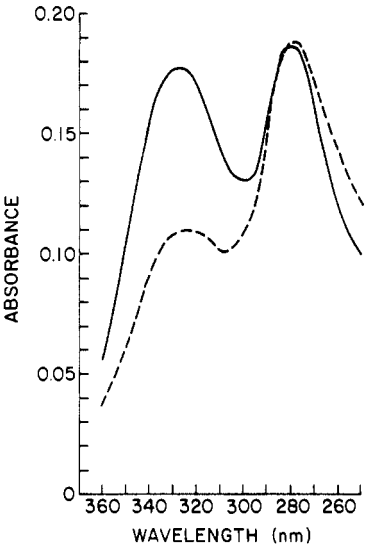


FIGURE 2: Absorption spectra of the retinol–RBP complex obtained after addition of retinol to either apo-RBP (—) or to the IEBA–RBP complex (---). In each case, excess retinol was added to the protein solution, followed by incubation for 1 h at 37 °C and then by gel filtration through Sephadex G-25 (see text for further details).

Table II: Binding of [^{3}H]IEBA to RBP and Subsequent Displacement by Retinol

incubation time (h)	^{3}H-labeled ligand bound to RBP[a] (mol/mol)	ligand bound to RBP after retinol addition (mol/mol)	
		^{3}H-labeled ligand	retinol[b]
2.5	0.73	0.20 ± 0.03	0.75 ± 0.02
5	0.89	0.44 ± 0.04	0.55 ± 0.03
10	0.95	0.69 ± 0.03	0.43 ± 0.02
18	0.94	0.69 ± 0.06	0.35 ± 0.02
24	1.25	0.75 ± 0.06	0.35 ± 0.03

[a] Before addition of retinol. [b] Values listed are uncorrected for IEBA absorbance at 280 nm.

increasing the subsequent inhibition of retinol binding. As shown in Table II (second column from left), the moles of ^{3}H-labeled ligand per mole of RBP increased slightly with time, and after 24 h the amount of ligand present slightly exceeded the available specific binding sites, probably due to a small amount of nonspecific binding.

With increasing time of incubation, between 2.5 and 10 h, progressively less of the bound ^{3}H-labeled ligand could be displaced by retinol, and a progressively increasing inhibition of the binding of retinol was observed. With incubation times of 18 or more hours, only 0.34–0.35 mol of retinol subsequently became bound to RBP (i.e., retinol binding was inhibited 60–65%) (Table II, right-hand column).

An experiment was carried out in which IEBA in 100-fold molar excess was added to the retinol–RBP complex to determine whether IEBA would displace retinol from the binding site. After 18-h incubation, 92% of the retinol was displaced from the binding site as determined from the absorption spectrum. This further demonstrates that IEBA occupies the retinol binding site of RBP.

Extraction of Noncovalently Bound Ligand. For a further investigation of whether the radioactive ligand that was not displaced by retinol was covalently bound to RBP, the RBP solutions from the study summarized in Table II were subjected to total lipid extraction with chloroform–methanol. Radioactivity (^{3}H) that was extractable presumably represented IEBA noncovalently bound to RBP; it was assumed that

Table III: [^{3}H]IEBA Extracted from RBP after Displacement by Retinol

incubation time (h)	[^{3}H]IEBA extracted (as % of total bound ^{3}H)	ligand covalently bound to RBP[a] (mol/mol)	ligand bound to RBP before extraction[a] (mol/mol)	
			retinol	total ligand[b]
0			0.92 ± 0.03	0.92 ± 0.03
2.5	23 ± 4	0.16 ± 0.03	0.77 ± 0.02	0.93 ± 0.03
5	22 ± 2	0.37 ± 0.01	0.59 ± 0.04	0.96 ± 0.04
10	24 ± 3	0.61 ± 0.04	0.49 ± 0.07	1.10 ± 0.04
18	20 ± 5	0.66 ± 0.04	0.36 ± 0.04	1.03 ± 0.04
24	12 ± 2	0.76 ± 0.10	0.41 ± 0.02	1.17 ± 0.11

[a] Values listed have been corrected for IEBA absorbance at 280 nm. [b] Sum of covalently bound [^{3}H]IEBA + bound retinol.

radioactivity (^{3}H) that could not be extracted represented IEBA covalently bound to RBP. As shown in Table III (second column from the left), only 12–24% of the radioactivity remaining associated with RBP after retinol addition could be extracted from the protein. In contrast (as a control for this experiment), 96% of the radioactivity present in a sample of [^{3}H]retinol complexed to RBP could be extracted into chloroform. These extraction data were used to calculate the amount of IEBA covalently bound to RBP (in each sample); these values, in turn, were used, for each sample, to correct the 280-nm protein absorbance of the IEBA–RBP complex for IEBA absorbance. The moles of covalently bound IEBA per mole of RBP calculated by using the corrected protein absorbance values are given in the third column of Table III. After 10 h of incubation, 0.61 mol of [^{3}H]IEBA was estimated to be covalently bound per mol of RBP. The corrected values for moles of retinol bound per mole of RBP are shown in the fourth column of Table III. Finally, the right-hand column of Table III shows the sum of the moles of covalently bound IEBA and of bound retinol per mole of RBP. It appears from the data in Table III that longer incubation times were associated with the occurrence of a little nonspecific covalent binding of IEBA to the protein. Thus, at the longest time periods studied, the moles of labeled retinoid per mole of RBP showed a slightly greater increase than would have been expected from the reduction in retinol binding, giving a slightly increasing total ligand to RBP ratio with increasing incubation time.

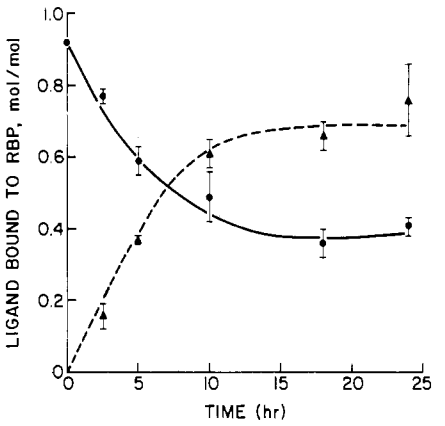


FIGURE 3: Inhibition of retinol binding to [^{3}H]IEBA–RBP with increased incubation time of the [^{3}H]IEBA–RBP complex. The two curves show the effects of time (abscissa) on (i) the amount of [^{3}H]IEBA remaining bound to RBP after displacement by addition of excess retinol (▲---▲) and (ii) the amount of retinol becoming bound to RBP under these conditions (●—●). The amount of bound ligand, in each case, is given as moles per mole of RBP. All values have been corrected for the absorbance at 280 nm of covalently bound IEBA.

Discussion

The experiments reported here describe the synthesis of three radioactive retinol analogues containing a reactive bromoacetyl group that were prepared as potential retinoid affinity labels for the retinol binding site of human plasma RBP. One of these compounds (β-ionylideneethyl bromoacetate, IEBA) appeared to be an effective affinity label for the retinol binding site of RBP. Three lines of evidence support this conclusion. First, after the formation of 1:1 molar complex between [^{3}H]IEBA and RBP, a large proportion of the radioactive retinoid could not be displaced from RBP with excess retinol. In contrast, with the other two radioactive retinoids (IBA and RBA) almost all of the retinoid bound to RBP could be displaced with retinol. Second, the binding of retinol to RBP, when excess retinol was added to the IEBA–RBP complex, was inhibited to an extent that was equivalent to the amount of [^{3}H]IEBA that was not displaced from RBP by retinol. Third, radioactive retinoid could not be extracted from RBP by a total lipid extraction. Taken together, these three lines of evidence strongly suggest that the ligand was bound in the retinol binding site of RBP and was attached to the protein in a covalent manner.

Inhibition of retinol binding increased with increased formation time of the IEBA–RBP complex (Figure 3), despite the fact that the radioactive retinoid was combined with RBP in a 1:1 molar ratio by 2.5 h. This indicates that a reversible (noncovalent) IEBA–RBP complex existed prior to irreversible (covalent) modification. We suggest that the bromoacetyl group reacts with a nucleophilic functional group in or very closely adjacent to the retinol binding site of RBP and that this is a slow reaction which takes place after IEBA is reversibly bound. The 1:1 molar correspondence between loss of retinol binding and the amount of radioactive retinoid incorporated into the protein (Figure 3) provides good evidence that IEBA is a true affinity label and is specific for the retinol binding site of RBP.

The studies reported here provide information that could be used to explore the structure of the retinol binding site on RBP, by identifying areas in the primary structure of RBP that are in contact with the ligand and that comprise part of the binding domain. A radioactive label bound to one or more amino acid residues in the binding site of RBP would provide a means by which the involved amino acids may be identified. The results reported here suggest that it should be feasible to use IEBA to obtain such a preparation of affinity labeled RBP. Enzymatic digestion of labeled RBP followed by peptide mapping should provide one or more peptides containing the radioactive label. Isolation of the labeled peptides, followed by amino acid analysis, and if necessary sequence analysis, should, if successful, permit one to determine which amino acid residues were modified by the affinity label.

The structural features required for the binding of retinol and structurally related compounds to RBP appear to be fairly, but far from absolutely, specific. A large number of studies have explored the binding of a variety of retinoids and related compounds to apo-RBP (Goodman & Raz, 1972; Heller &

Horwitz, 1973; Horwitz & Heller, 1973, 1974a; Cogan et al., 1976; Hase et al., 1976). Changing the functional group of retinol from a hydroxyl group to a carboxylic acid does not significantly affect the binding of the retinoid to RBP. Retinaldehyde and retinyl acetate also bind to RBP, although some investigators have reported less (Goodman & Raz, 1972; Hase et al., 1976), whereas others have reported similar binding (Horwitz & Heller, 1973; Cogan et al., 1976) of these compounds to RBP, as compared to the binding of retinol. The discrepancy between these results may be due to the various experimental methods employed and the different criteria used to assay for binding activity. The long-chain retinyl ester, retinyl palmitate, showed virtually no affinity for RBP (Hase et al., 1976; Cogan et al., 1976); retinoic acid esters also do not appear to form a complex with RBP (Hase et al., 1976). From the results of these studies it can be concluded that the functional groups affecting the binding of retinyl compounds can be listed tentatively in order of decreasing affinity: retinol and retinoic acid have the greatest affinity for RBP, followed by retinaldehyde and retinyl esters, with long-chain retinyl esters and retinoic acid esters being the least likely to bind.

The 13-cis and 11-cis,13-cis isomers of retinol bound to RBP somewhat less well than did the all-trans isomer (Goodman & Raz, 1972). In studying the binding of retinaldehyde to RBP, it was observed that the 13-cis, 11-cis, and 9-cis isomers bound virtually as well as did the all-trans isomer (Horwitz & Heller, 1973). It hence appears that the retinol binding site is not very sensitive to configurational changes in the side chain of the retinyl ligand.

The unsuccessful attempts to form an RBP complex with compounds such as phytol and a number of other terpenoids (Goodman & Raz, 1972; Hase et al., 1976) indicate that the cyclohexene ring structure is probably necessary for binding. However, experiments with β-ionone and β-ionylideneacetic acid (Hase et al., 1976), as well as experiments described in this paper, demonstrate that the complete polyene side chain of retinol is not essential for binding to RBP.

The three compounds described in this paper comprise a homologous series of retinoids with varying chain lengths. The longest of the three compounds (retinyl bromoacetate, RBA) bound poorly to RBP, whereas the other two compounds bound well. The poor binding of RBA may be due in part to the increased length of the ligand. After formation of the reversible RBA–RBP complex, no covalent bonding took place, indicating that the bromoacetate group of the ligand was not sufficiently close to a reactive amino acid for the reaction to occur.

The shortened side chain of β-ionyl bromoacetate (IBA) allowed a 1:1 molar reversible binding with RBP to occur, but this compound also did not covalently bond to RBP. The failure of IBA to react with an amino acid residue in the retinol binding site may be explained by the position of the reactive methylene group of its bromoacetyl moiety, which corresponds to carbon 12 of retinol. This area of the ligand is most likely close to unreactive hydrophobic residues of the protein. In contrast, the covalent reaction that occurred between IEBA and RBP can be attributed to the location of the reactive group of IEBA with respect to the parent compound. The methylene carbon of the bromoacetyl group of IEBA corresponds roughly to carbon 14 of retinol and may be sufficiently close to the polar end of the binding site to form a covalent bond with an amino acid residue which normally interacts with the hydroxyl of retinol.

Detailed information about the retinol binding site of RBP would be of biological as well as chemical interest. Previous work has shown that the ligand retinol plays an important role in the regulation of RBP secretion from the liver and in the maintenance of plasma RBP levels (Muto et al., 1972; Smith et al., 1973). The mechanism whereby retinol regulates the secretion of RBP is not known. One possibility is that the interaction of retinol with the binding site leads to an induced conformational change in the protein which may initiate the process of secretion. Structural information about the binding site would help to explore this and other possibilities.

Finally, the possibility exists that IEBA (or one of the other retinoid bromoacetates described here) might also be able to serve as an affinity label for the retinoid binding sites of one of the intracellular retinoid-binding proteins, cellular retinol-binding protein (CRBP) or cellular retinoic acid binding protein (CRABP) [see Chytil & Ong (1978, 1979) and Goodman (1981) for reviews and references]. During the past few years there has been expanding interest in the properties and possible functions of these binding proteins, and a good deal of information about them is now available. If either of these two intracellular proteins binds any of the retionid bromoacetates described here, the possibility of using these compounds as affinity labels for the cellular-binding proteins would merit consideration.

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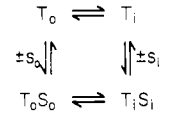
Changes in the Intrinsic Fluorescence of the Human Erythrocyte Monosaccharide Transporter upon Ligand Binding†

Frank R. Gorga‡ and Gustav E. Lienhard*

ABSTRACT: The effect of ligands on the tryptophan fluorescence of the purified monosaccharide transporter from human erythrocytes has been investigated. Cytochalasin B, D-glucose, and ethylideneglucose quench the fluorescence of the protein at longer wavelengths by 17%, 13%, and 8%, respectively. Propyl glucoside, another ligand, has no effect on the protein fluorescence. Values of the dissociation constants for cytochalasin B, D-glucose, and ethylideneglucose were determined from the concentration dependence of fluorescence change; these agree with the values obtained from the effects of these compounds upon the binding of [^{3}H]cytochalasin B measured by equilibrium dialysis. There is no correlation between the effect of each ligand on the fluorescence of the transporter and the conformational state expected for its complex on the basis of other evidence. The fact that the quenching is greatest at longer wavelengths suggests that an exposed tryptophan residue(s), possibly located at the ligand binding sites, is the perturbed one.

Most of the information concerning the steady-state kinetics (Geck, 1971; Regen & Tarpley, 1977; Foster & Jacquez, 1976), inhibitor specificity (Barnett et al., 1975), and substrate binding properties (Gorga & Lienhard, 1981) of the monosaccharide transporter of the human erythrocyte is consistent with an alternating conformation model for transport (Vidaver, 1966; Baldwin & Lienhard, 1981). According to this model, the transporter alternates between a conformation in which there is a substrate binding site at the external surface (T_o) and a conformation in which there is a site at the cytoplasmic surface (T_i). Translocation of D-glucose and other substrates occurs when the occupied transporter (T_oS_o, T_iS_i forms) undergoes the conformational change. The postulated species and reactions are summarized in the following scheme:



The monosaccharide transporter has been shown to be a transmembrane protein that does not undergo complete rotation across the bilayer (Baldwin et al., 1980); consequently, the transition between the two conformers is envisioned as a limited structural rearrangement occurring within a protein that has a largely fixed orientation in the bilayer.

Now that the monosaccharide transporter is available in purified form (Sogin & Hinkle, 1980; Baldwin et al., 1980; Baldwin & Baldwin, 1981), it should be possible, if the alternating conformation model is correct, to obtain direct evidence for the occurrence of the two conformations and to determine the values of the rate and equilibrium constants for their interconversion, in both the unoccupied and occupied states. As an initial step in this project, we describe herein changes in the intrinsic fluorescence of the transporter that occur upon ligand binding.

Experimental Procedures

Materials. 4,6-*O*-Ethylidene-α-D-glucose (ethylideneglucose) was purchased from Aldrich; *n*-propyl β-D-glucopyranoside (propyl glucoside) was synthesized after the method of Barnett et al. (1975), as described previously (Gorga & Lienhard, 1981). Both of these sugars were purified by chromatography on cellulose and by treatment with charcoal (Gorga & Lienhard, 1981). D-Glucose (ACS grade from Fisher), L-glucose (Pfanstiehl), cytochalasin B (Aldrich), [^{3}H]cytochalasin B (New England Nuclear), and 21,22-dihydrocytochalasin B (a generous gift from Dr. D. C. Aldridge of ICI Ltd.) were each used as obtained.

Transporter. The glucose transporter was purified from human erythrocyte membranes by a modification (Baldwin & Baldwin, 1981; S. A. Baldwin, J. M. Baldwin, and G. E. Lienhard, unpublished results) of the method described by Baldwin et al. (1980). In this procedure, erythrocyte membranes that have been depleted of peripheral proteins are extracted with 1.35% octyl glucoside, and the transporter is isolated by chromatography of the extract on DEAE-cellulose. The transporter, which, together with a portion of the erythrocyte lipids, is not adsorbed by the DEAE-cellulose, is reconstituted into bilayers through the removal of the detergent by dialysis, without the addition of exogenous phospholipid. This method results in a preparation containing about 130 μg of protein and 400 μg of erythrocyte phospholipid per mL in 100 mM NaCl/50 mM Tris-HCl/1 mM EDTA, pH 7.4, which is frozen in liquid nitrogen and stored at −70 °C.

We have examined the form of the purified, reconstituted transporter by gel filtration on a calibrated column of Bio-Gel

† From the Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755. *Received October 20, 1981.* This work was supported by Grant GM22996 from the National Institutes of Health and by a fellowship from the Albert J. Ryan Foundation to F.R.G.

‡ Present address: Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA 02138.

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