Modification of Tryptophan Residues in Retinol-binding Protein and Prealbumin with 2-Hydroxy-5-nitrobenzyl Bromide

EFFECTS OF THE MODIFICATION ON THE PROTEIN-RETINOL AND PROTEIN-PROTEIN INTERACTION

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SUMMARY

Human retinol-binding protein (RBP) and prealbumin were labeled with 2-hydroxy-5-nitrobenzyl bromide (HNB-Br) at pH 5.5 using 20- to 100-fold molar excess of the label over tryptophan in each protein. In prealbumin, only 1 to 1.7 tryptophan residues out of 8 were modified under these conditions. This modification did not alter the capacity of prealbumin to bind to retinol-RBP at physiological ionic strength.

When native retinol-RBP and apo-RBP were labeled with HNB-Br, 1.1 to 2.1 out of the total of 4 tryptophan residues were modified. This modification did not affect the ability of RBP to bind to retinol, or the ability of apo-RBP to form the retinol-RBP complex upon addition of retinol. The modified retinol-RBP and reconstituted apo-RBP, however, did not form a complex with prealbumin at physiological ionic strength.

HNB-Br modification of retinol-RBP-prealbumin complex resulted in 0.7 tryptophan residue being modified in each protein. This modification did not affect the properties of either retinol-RBP, prealbumin, or the interaction between the two proteins. It is concluded, therefore, that 1 of the tryptophan residues modified in native and apo-RBP is involved in the binding between prealbumin and retinol-RBP.

In addition, both RBP and prealbumin are capable of binding noncovalently to the HNB chromophore under the conditions of the modification used.

Retinol is transported in the plasma by a specific binding protein (1). In the plasma, one molecule of retinol-binding protein (RBP) is bound to one molecule of prealbumin (1-5). The binding between retinol-RBP and prealbumin is equivalent and highly specific (1, 3-7). We have shown recently that the functional group of the retinyl moiety will affect the interaction between prealbumin and RBP. Thus, while retinol-RBP binds to prealbumin at physiological ionic strength, retinal-RBP will not bind to prealbumin (6, 7). Raz et al. (4) have shown previously that reductive alkylation of disulfide bonds as well as iodination of retinol-RBP and prealbumin also will affect the retinol to RBP interaction and retinol-RBP to prealbumin interactions.

To gain further information on the interaction between the chromophoric group and RBP and between RBP and prealbumin, we have used 2-hydroxy-5-nitrobenzyl bromide (HNB-Br) to selectively modify tryptophan residues in both prealbumin and RBP.

EXPERIMENTAL PROCEDURE

Materials—The preparation of retinol-RBP, prealbumin, and apo-RBP from human plasma was described previously (6). The retinol-RBP-prealbumin complex had an $A_{340}:A_{280}$ ratio of about 2.4 in different preparations. Free all-trans-retinol-RBP and prealbumin were prepared from the complex by dissociation in 1 mM Tris-HCl buffer, pH 9.0, and separated from each other by gel filtration chromatography on Sephadex G-100 in the same buffer. All-trans-retinol-RBP has an $A_{340}:A_{280}$ ratio of 0.98 to 1.02 in different preparations. Apo-RBP was between 97 and 100% retinol free. Fresh solutions of all-trans-retinol were obtained as previously described (6). HNB-Br was obtained from Pierce Chemical Co.

Protein Modifications—These were performed as described by Horton and Koshland (8). Typically, 1 to 4 mg of HNB-Br were dissolved in 20 to 50 &mu;L of acetone. The appropriate amount of HNB-Br was added to 1 ml of the protein solution in 1 mM sodium acetate buffer, pH 5.5, at 20°. The modified protein then was passed through a column of Sephadex G-50 (fine, 20 X 1.5 cm) equilibrated with the same buffer to remove the excess label.

Determination of Number of Tryptophan Residues Modified—The number of moles of HNB bound per mole of protein was estimated by bringing the modified protein solution to pH > 12 with NaOH and then measuring the absorbance at 410 nm using $e = 18,450$ M$^{-1}$ cm$^{-1}$ as described by Horton and Koshland (8). The number of modified tryptophan residues was determined by amino acid analysis using the Beckman analyzer 120C after hydrolysis with 3 N p-toluenesulfonic acid according to Liu and Chang (9).

Binding of Retinol-binding Protein to Prealbumin—Binding studies were performed by gel filtration chromatography on a column (1.5 X 89 cm) of Sephadex G-100 equilibrated in 0.033 M sodium phosphate buffer containing 0.1 M NaCl either at 20 or 4° as previously described (6).

Spectroscopic Studies—Absorption and circular dichroism were measured as described previously (6).

Protein Concentrations—These were determined from the
Effect of varying HNB-Br to tryptophan ratio on incorporation of HNB into retinol-RBP

<table>
<thead>
<tr>
<th>Moles excess of HNB-Br over tryptophan</th>
<th>Estimated number of moles of HNB bound per mole of retinol-RBP from absorbance at 410 nm&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of modified tryptophan residues from amino acid analysis&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>1.6</td>
<td>1.1</td>
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<tr>
<td>30</td>
<td>2.0</td>
<td>1.9</td>
</tr>
<tr>
<td>80</td>
<td>2.2</td>
<td>2.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> This number represents the total estimated moles of HNB bound covalently and noncovalently to retinol-RBP after passing the HNB-protein through a column of Sephadex G-25 (see "Noncovalent Binding of HNB to RBP and Prealbumin" under "Results").

<sup>b</sup> Number of tryptophan residues modified was determined by amino acid analysis as described under "Experimental Procedure." The number of tryptophan residues in native retinol-RBP was 3.7 (all other residues were similar to previously published results (1)). The number of tryptophan modified was calculated as the difference between tryptophan residues observed in native and modified retinol-RBP.

absorbance 280 nm using a value of ε<sub>280</sub> = 46,000 for retinol-RBP, ε<sub>280</sub> = 76,000 for prealbumin, and ε<sub>280</sub> = 40,400 for apo-RBP (6).

RESULTS

HNB-Br Modification of Native Retinol-RBP—When native retinol-RBP was modified with 30-fold molar excess of HNB-Br over tryptophan at pH 5.5 and the excess HNB-Br removed by gel filtration, the spectrum of the resulting product was as shown in Fig. 1. The A<sub>430</sub>: A<sub>410</sub> ratio of 0.82 in the modified retinol-RBP as compared to 1.0 in native retinol-RBP is to be expected since at pH 5.5 HNB-Br absorbs maximally around 330 nm, which is where the retinol absorbs in native retinol-RBP. As judged from the amino acid analysis, 1.0 tryptophan residues were modified.

Modification of native retinol-RBP with 20- and 80-fold molar excess of HNB-Br over tryptophan resulted in 2.1 and 1.1 tryptophan residues being modified (Table I).

Circular Dichroism of HNB-RBP—The circular dichroism spectra of the modified protein between 420 and 260 nm were identical with that of native RBP (6), suggesting that the modification did not alter significantly the interaction between the chromophore and the protein. In addition, the HNB chromophore did not seem to exhibit induced optical activity (see also below, under "HNB Modification of Apo RBP").

Interaction between HNB-Retinol-RBP and Prealbumin—As was shown previously, retinol-RBP will form a complex with prealbumin that will not dissociate at physiological ionic strength (1, 4, 6). However, when HNB-retinol-RBP was incubated with prealbumin at physiological ionic strength and then passed through a column of Sephadex G-100, the prealbumin was found to be dissociated from HNB-retinol-RBP. The first peak to emerge from the column was that of prealbumin by itself with its expected elution volume; the second peak was that of HNB-retinol-RBP with the elution volume being that of native retinol-RBP. No retinol was lost from the HNB-modified retinol-RBP after passing through the column, as judged from the A<sub>430</sub>: A<sub>410</sub> ratio.

We have observed previously that when solid NaCl was added to a mixture of native retinol-RBP and prealbumin in a low ionic strength buffer, the absorption spectrum of the chromophore at 330 nm and the CD spectrum of the mixture at 250 to 300 nm showed a hyperchromic effect (6). However no hyperchromic effect was observed under the same conditions when HNB-retinol-RBP was incubated with prealbumin.

HNB Modification of Apo-RBP—The absorption spectra of apo-RBP modified with 20-fold molar excess of HNB-Br over tryptophan is shown in Fig. 1. Since apo-RBP does not have any absorption at 320 nm because of the removal of the retinol chromophore (see Fig. 2 in Ref. 6), the absorption at 320 and 410 nm seen in Fig. 1 is due solely to the HNB chromophore. As judged from the amino acid analysis, 1.5 tryptophan residues were modified in this case. When the amount of HNB was increased to 80-fold molar excess per mole of protein tryptophan, amino acid analysis showed that 1.2 tryptophan residues were modified, while from spectroscopic measurements at 410 nm upon addition of NaOH, 2.5 moles of HNB per mole of apo-RBP were calculated to be bound (Table II).

Circular Dichroism of HNB-modified Apo-RBP—The CD spectrum of the modified apo-RBP was identical with that of unmodified apo-RBP (Fig. 2). Thus, even though the absorption spectra of HNB-modified apo-RBP shows a strong band at 320 nm, no CD band is observed at this wavelength. Like the HNB-modified retinol-RBP, the HNB chromophore in the apo RBP does not exhibit extrinsic optical activity.

Reconstitution of HNB-modified Apo-RBP with All-trans-Retinol—When the modified apo-RBP was incubated with all-trans-retinol, the CD spectrum was indistinguishable from that of
Fig. 2. Comparison between the CD spectra of apo-RBP and HNB-modified apo-RBP, and between reconstituted HNB-modified apo-RBP and native retinol-RBP. Curve 1, 37 μM apo-RBP; Curve 2, 39 μM HIND-modified apo-RBP; Curve 3, 12 μM reconstituted HNB-modified apo-RBP. O—O, SD spectrum of native prealbumin. BL, base-line. Path length in all experiments was 10 mm.

Table III

<table>
<thead>
<tr>
<th>Moles excess of HNB-Br over tryptophan</th>
<th>Estimated number of moles of HNB bound per mole of prealbumin from absorbance at 410 nm</th>
<th>Number of modified tryptophan found from amino acid analysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>40</td>
<td>2.6</td>
<td>1.1</td>
</tr>
<tr>
<td>100</td>
<td>1.5</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* This number represents the total estimated moles of HNB bound covalently and noncovalently to prealbumin after passing the HNB-prealbumin complex through a column of Sephadex G-25 (see “Noncovalent Binding of HNB to RBP and Prealbumin” under “Results”).

† Number of tryptophan residues modified was determined by amino acid analysis as described in “Experimental Procedure.” The number of tryptophan residues in native prealbumin was 7.9 (all other residues were similar to previously published results (3)). The number of tryptophan modified was calculated as the difference between tryptophan residues observed in native and modified prealbumin.

Fig. 3. Comparison between the CD spectra of HNB-modified prealbumin and native prealbumin. —, spectrum of 24 μM prealbumin where 3 tryptophan residues were modified by HNB-Br. O---O, CD spectrum of native prealbumin. All spectra in 2 mm Tris-HCl buffer, pH 9. Path length, 10 mm.

Table IV

<table>
<thead>
<tr>
<th>Modified fraction</th>
<th>Estimated number of moles of HNB bound vs. mode of protein from absorbance at 410 nm</th>
<th>Number of modified tryptophan from amino acid analysis*</th>
<th>Binding between the modified fraction and the native protein*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prealbumin</td>
<td>1.2</td>
<td>0.7</td>
<td>+</td>
</tr>
<tr>
<td>RBP</td>
<td>0.9</td>
<td>0.7</td>
<td>+</td>
</tr>
</tbody>
</table>

* Same as Footnote a in Tables I and III.

† Same as Footnote b in Tables I and III.

‡ + means that when the modified prealbumin fraction was mixed with native RBP, or when the modified RBP fraction was mixed with native prealbumin at physiological ionic strength and then applied to Sephadex G-100 column, the two proteins formed a complex with an elution volume which was indistinguishable from the native complex.

However, unlike retinol-RBP and apo-RBP, the HNB chromophore produced an induced positive CD band peaking at 390 nm (Fig. 3).

Interactions between HNB-modified Prealbumin and Native Retinol-RBP—We define binding between RBP and prealbumin as a molecular complex that can be isolated by gel filtration chromatography or electrophoresis and that is different from either free retinol-RBP or prealbumin (5). Each of the HNB-modified prealbumin preparations shown in Table III was able to bind to native retinol-RBP at physiological ionic strength as judged by gel filtration chromatography. This binding was indistinguishable from the normal binding between native prealbumin and native retinol-RBP.

HNB Modification of Complex Retinol-RBP-Prealbumin—Since at physiological ionic strength, retinol-RBP forms a 1:1 complex with prealbumin, it was of interest to modify the complex, the rationale being that in the complex fewer tryptophan residues will be modified. Amino acid analysis showed that 0.7 tryptophan residues were modified in prealbumin and 0.7 in retinol-RBP, as determined after dissociation of the two proteins (Table IV). When the modified retinol-RBP-prealbumin complex was chromatographed on the Sephadex G-100 column at physiological ionic strength, it appeared as a single peak at the elution volume identical to that of unmodified retinol-RBP-prealbumin complex. Thus, in this case the modified prealbumin and the modified retinol-RBP did form a complex at high ionic strength that did not dissociate.
When the modified retinol-RBP-prealbumin complex was chromatographed in low ionic strength buffer (2.0 mM Tris, pH 9), the modified prealbumin dissociated from the modified RBP as in the case with the native complex. In addition, the modified prealbumin (after dissociation from the modified RBP) was tested for its ability to form a complex with native retinol-RBP, and the HNB-modified retinol-RBP (after dissociation from the modified prealbumin) was tested for its ability to form a complex with native prealbumin at physiological ionic strength. In both cases a complex identical to that between the two native proteins was formed, as judged by gel filtration chromatography (Table IV).

**Noncovalent Binding of HNB to RBP and Prealbumin**—As seen in Tables I through III, in several experiments, the number of tryptophan residues, modified as determined by amino acid analysis, did not agree well with the estimated number of moles of HNB bound to the protein. Since the HNB moiety is relatively hydrophobic, and since both RBP and prealbumin transport hydrophobic groups, the possibility arose that some HNB was noncovalently bound to these proteins even after passing through the gel filtration column. An example of an absorption spectrum of an HNB-modified prealbumin after passing through a column of Sephadex G-25 (20 × 1.5 cm) is seen in Fig. 4. The estimated number of bound moles of HNB in this preparation from the absorption measurements at 410 nm was 1.8. However, when the protein was precipitated in 90% ethanol, the ethanolic supernatant contained 40% of the HNB chromophore, presumably HNB-OH (see Fig. 4). Upon redissolving the protein, a higher ratio of A_{280}/A_{10} is observed (Fig. 4), resulting in 1.1 tryptophan residue being modified. This number is expected since 40% of the total chromophore was recovered in the supernatant.

In another experiment with retinol-RBP, where a discrepancy was found between the number of tryptophan residues modified as determined by amino acid analysis and spectroscopy, it was determined by solvent extraction that the excess HNB chromophore was noncovalently bound to the retinol-RBP.

**DISCUSSION**

The HNB-Br modifications of prealbumin and RBP were performed under relatively mild conditions (pH = 5.5), where both proteins behave and interact as in their native states (4). Because free -SH groups were not detected in either RBP or prealbumin (4), the HNB-Br is expected to modify tryptophan residues only. The results, however, show that, in addition to the modified tryptophan residues, the HNB chromophore is sometimes capable of binding noncovalently to both proteins. Since both proteins transport hydrophobic molecules, they may have additional nonspecific binding sites that can carry the hydrophobic HNB moiety. Therefore, in order to get a better colorimetric estimation of the number of tryptophan modified, it was necessary to precipitate the proteins and to extract the noncovalently bound HNB chromophore with ethanol (11).

Prealbumin was found to be less susceptible to modification by HNB-Br than retinol-RBP since only 1 to 1.7 tryptophan residues were modified out of a total of 8. Presumably most of the tryptophan residues are not accessible to HNB-Br under these conditions. The characteristic sharp CD band of prealbumin at 290 nm (Fig. 3) which arises most probably from the 0-0 L_{ab} transition of a tryptophanyl moiety (or moieties) was not altered when 1 to 2 tryptophan residues were modified (12). Thus, this 290-nm CD band must arise from the tryptophan residues which are not available to the HNB-Br. The modification of the tryptophan residues in prealbumin did not alter the capacity of prealbumin to bind to retinol-RBP at physiological ionic strength. Similarly, Raz et al. (4) found previously that reductive alkylation of one of the two disulfide bonds in prealbumin as well as iodination of prealbumin with approximately 4.6 atoms of iodine per molecule did not alter prealbumin binding affinity for retinol-RBP.

In the case of retinol-RBP and apo-RBP, 1.1 to 2.1 of the 4 tryptophan residues were modified. However, this modification did not alter the capacity of retinol-RBP and apo-RBP to bind retinol. On the other hand, this modification did interfere with the ability of retinol-RBP to bind to prealbumin. Therefore, while the modified tryptophan residues are not involved in the binding of retinol to RBP, they are involved in the binding of retinol-RBP to prealbumin.

The modification of retinol-RBP-prealbumin complex which resulted in about 1 tryptophan residue being modified in each protein did not seem to affect the properties of either retinol-RBP, prealbumin, or the interaction between them. Since not more than 2 tryptophan residues were modified in apo-RBP and in native retinol-RBP under the various concentrations of HNB-Br tried and since these modified proteins were not capable of binding to prealbumin, it seems reasonable to assume that only 1 tryptophan residue is involved in the complex formation between prealbumin and retinol-RBP. This tryptophan residue seems to be protected from the HNB-Br when retinol-RBP is bound to prealbumin.

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**REFERENCES**

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