A Cortisol-induced Charge Difference in Desialylated Human Transcortin Detected by Isoelectric Focusing*

(Received for publication, August 16, 1971)

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SUMMARY

Human desialylated transcortin was found to be microheterogeneous by isoelectric focusing. All samples tested showed two cortisol-binding components. Liganded with cortisol these components are isoelectric at pH 5.2 and 5.4. In the unliganded form, however, they eluted at pH 5.4 and 5.6, respectively. This cortisol-controlled transition was reversible in both directions. Isolated components containing trace amounts of [3H]cortisol accumulate the steroid in the position of the corresponding liganded form at the anodic side of the unliganded binder. Electrofocusing of desialylated plasma containing [3H]cortisol results in a cortisol-controlled transition being carried out as above. Electrofocusing can profitably be used to study ligand-protein interactions and that radioligand detection is not always an adequate method to localize a macromolecular binding agent.

EXPERIMENTAL PROCEDURE

Materials

Ampholine carrier ampholytes, covering 2 pH units, were obtained from LKB as 40% solutions. Neuraminidase, type VI, was purchased from Sigma. Dialysis tubings (A. H. Thomas Co.) were boiled twice in distilled water and thoroughly rinsed before use. All solutions were made with glass-redistilled, deionized water. All other chemicals were analytical reagent grade.

Different lots of [1,2-3H]cortisol with an average specific activity of about 30 Ci per mmole were obtained from New England Nuclear Corp. Preparations of a radiochemical purity lower than 95% were subjected to paper chromatography.

Serum or plasma was obtained from normal adults and was stored at -20°C.

Methods

Isoelectric Focusing—All experiments were carried out with the LKB model 8101 column (110-ml capacity). In a first series of separations constant sucrose gradients (from 46 to 4%) were prepared manually by layering 22 4.6-ml fractions obtained by mixing appropriate volumes of a 0.8% ampholyte solution and of a solution containing 46% sucrose and 0.8% ampholytes. An appropriate volume of protein sample was introduced in place of the corresponding volume of less dense solution used to prepare one or several consecutive fractions. Electrofocusing was carried out at 4°C for 45 hours. An initial potential of 600 volts was maintained for 2½ hours. The voltage was then increased to 1000 volts. At the end of the experiment the column was drained at a constant flow rate of 1.4 ml per min by replacing the gradient with water pumped through the lower gas outlet. Fractions of 2.8 ml were collected.

In another series of separations previously formed pH gradients were used. Electrofocusing of a sucrose gradient (constructed as above) containing 10% ampholytes but no protein was carried out at 4°C for 48 hours with a maximum power output of 3 watts. Fractions of 4.2 ml were collected and their pH determined. To construct the final column, 0.4 ml aliquots of 22 successive fractions, constituting the central shallow pH gradient portion, were diluted with 4.2, 4.0, . . . 0.2, 0.0 ml of 46% sucrose solution, respectively, and further brought to a final volume of 4.6 ml with water. In one of the mixtures water was partially replaced by the protein solution. The pH was readjusted, if necessary, to the value measured before addition. Electrofocusing was carried out as above.

Preparations—Steroid-free plasma was prepared by incubation with powdered charcoal (9). By electrofocusing such a treatment was shown not to affect the isoelectric behavior of asialotranscortin. Removal of sialic acids was performed by dialyzing 10 ml of plasma and 0.2 mg of neuraminidase at room temperature for 24 hours against 250 ml of a 0.05 M sodium acetate buffer, pH 5.8, containing 0.17 M sodium chloride and 0.02% sodium azide. The buffer was then renewed and the
same amount of neuraminidase was added to the dialysis bag. After another 24 hours the protein solutions were dialyzed overnight in the cold against 100 volumes of distilled water. Some precipitate was removed by centrifugation. This did not cause any loss of binding activity. The resulting preparation contained less than 2% of the sialic acid originally present as determined by the method of Warren (10).

Other Techniques—The pH value of eluted fractions was measured at 4°C with a Radiometer type pHM 26 pH meter equipped with a Radiometer GK 2301 C combined electrode, standardized with Radiometer standard buffer (pH 6.50 ± 0.02 at 20°C). The cited pH values are those measured. Since the average ΔpH value between two subsequent fractions was 0.08, these values might be slightly different from the actual isolectric points.

Cortisol-binding activity was measured at 4°C by equilibrium dialysis for 48 to 72 hours under continuous rotation. Each dialysis bag contained 5 ml of diluted protein solution. The ratio of inner to outer volumes was 1:3. To study the effect of various parameters (pH, ionic strength, etc.) individual bags were dialyzed against the appropriate solutions. To localize binding activity in the fractions from the electofocusing columns multiple equilibrium dialysis (11) was performed in a phosphate buffer solution ampholine solution. To this end steroid-free plasma samples were treated with neuraminidase and focused in pH gradients containing either a uniform cortisol concentration (6 × 10^-8 M) or no cortisol. In a gradient without cortisol two cortisol-free binding components were eluted. Since cortisol exhibits moderate cortisol binding in this pH region (cf. Fig. 1), a possible effect of cortisol on its isoelectric position could be investigated. To this end steroid-free plasma samples were treated with neuraminidase and focused in pH gradients containing either a uniform cortisol concentration (6 × 10^-8 M) or no cortisol. In a gradient without cortisol two cortisol-free binding components were eluted. Also a minor peak was observed for both components when the same sample was focused in a gradient with cortisol, the minor component being eluted at pH 5.17 and the major at pH 5.37 (Fig. 2, dashed lines). An anodic shift of about 0.2 pH unit was observed for both components when the same sample was focused in a gradient with cortisol, the minor component being eluted at pH 5.38 and the major at pH 5.58 (Fig. 2, full lines). Since the excess of cortisol will keep the binding components in their cortisol-bound forms, these experiments show that liganded components have lower isoelectric points than the corresponding unliganded forms. These results were confirmed on three other plasma samples analyzed under identical conditions (Table 1).

Effects of pH, Ampholyte, and Sucrose on Cortisol-binding Activity—Because of the particular conditions of the focusing technique, the effects of pH, ampholyte, and sucrose on the binding of cortisol to native and desialylated plasma were studied by equilibrium dialysis. As indicated in Fig. 1, the cortisol-binding activity strongly depends on the pH of the solution, confirming previous work on native plasma (12). Identical binding profiles were obtained for native and desialylated plasma at an ionic strength of 0.1 in acetate and phosphate buffers (Fig. 1, left). These results are in accordance with the finding that desialylation of transcortin does not affect the association constant (8). However, in solutions containing fractionated ampholyte at a final concentration of 0.5%, the binding profiles did not coincide (Fig. 1, right). Similar results were obtained in ampholyte solutions containing sucrose at a final concentration of 20%. Since the binding profiles did not diverge in phosphate buffers of ionic strength 0.005, this phenomenon is most probably to be ascribed to the low ionic strength of the focusing medium and not to an interaction of ampholyte or sucrose with the binding proteins.

Electrofocusing of Native Transcortin—Isoelectric focusing experiments designed to obtain the binding spectrum of native transcortin were unsuccessful. Although some cortisol-binding activity could be detected around pH 4, the recovery was low, probably due to irreversible destruction of transcortin in this pH region (12). Therefore, attempts were made with asialotranscortin which was expected to display a less acidic isoelectric point due to removal of negatively charged sialyl residues.

Electrofocusing of Asialotranscortin—Desialylation resulted in a cathodic shift of the binding activity to pH above 5 (cf. Figs. 2 and 4). Since asialotranscortin exhibits moderate cortisol binding in this pH region (cf. Fig. 1), a possible effect of cortisol on its isoelectric position could be investigated. When cortisol was added to the focusing medium and focused in pH gradients containing either a uniform cortisol concentration (6 × 10^-8 M) or no cortisol, the cortisol binding strongly depends on the pH of the solution, confirming previous work on native plasma (12). Identical binding profiles were obtained for native and desialylated plasma at an ionic strength of 0.1 in acetate and phosphate buffers (Fig. 1, left). These results are in accordance with the finding that desialylation of transcortin does not affect the association constant (8). However, in solutions containing fractionated ampholyte at a final concentration of 0.5%, the binding profiles did not coincide (Fig. 1, right). Similar results were obtained in ampholyte solutions containing sucrose at a final concentration of 20%. Since the binding profiles did not diverge in phosphate buffers of ionic strength 0.005, this phenomenon is most probably to be ascribed to the low ionic strength of the focusing medium and not to an interaction of ampholyte or sucrose with the binding proteins.

To ascertain the reversibility of this transition in both directions, isolated peak fractions were dialyzed against distilled water and refocused separately under conditions opposite to those of the first separation (Fig. 3). Refocusing in a gradient
with cortisol of initially unliganded minor and major components shifted their respective isoelectric points to those of the corresponding liganded forms (Fig. 3, a and b). Refocusing experiments of initially liganded minor and major peak fractions in a gradient without cortisol were performed on samples containing 3.6 ng of [3H]cortisol. The major part of the binding activity was now confined to the positions of the unliganded forms, the remainder being eluted together with [3H]cortisol in the positions of the corresponding liganded forms (Fig 3, c and d).

Electrofocusing of desialylated plasma containing endogenous cortisol and trace amounts of [3H]cortisol resulted in a similar but more pronounced separation of cortisol and binding activity. The binding patterns corresponded to those obtained under cortisol-free conditions (cf. Fig. 2) whereas [3H]cortisol was eluted at pH 5.10 ± 0.03 (n = 9), a position where almost no cortisol binding was observed. In the experiments with [3H]cortisol radioactive tailing was negligible. It should be noted that in these experiments binding material was allowed to migrate anodically toward its isoelectric position. Since free cortisol does not migrate in the electric field these experiments strongly suggest accumulation of cortisol due to interaction with binding material.

To substantiate this hypothesis desialylated plasma was applied at pH 4.68 in a previously formed pH gradient containing a uniform trace concentration of [3H]cortisol (6 × 10^{-10} M). As illustrated in Fig. 4, minor and major binding components were eluted at pH 5.44 and 5.63, close to the positions of unliganded components. The cortisol elution profile showed a dip around the application position, a pronounced peak at pH 5.11 but virtually no cortisol at higher pH. Since the major portion of the cortisol peak originated from the region of binding activity, the cortisol accumulation necessarily results from interaction with binding components.

**DISCUSSION**

Electrofocusing of desialylated plasma and localization of cortisol-binding activity in the eluate by means of multiple equilibrium dialysis resulted in a binding pattern consisting of a minor and a major component. Since the contribution of the albumin interaction to the cortisol-binding activity is very small, these activities are mainly, if not exclusively, due to the high affinity-binding properties of asialotranscortin. Although incomplete cleavage of sialyl residues as the cause of the observed heterogeneity seems an attractive explanation, other possibilities cannot be excluded.

To explain cortisol accumulation the following mechanism is proposed. As a consequence of the cortisol-induced charge difference (Fig. 2), unliganded and liganded components migrate with different speeds toward their respective isoelectric positions. During migration liganded components continuously dissociate. In case of anodic migration the freed cortisol can be carried further by slower moving unliganded binding material. This process goes on until all cortisol has been accumulated in the position of the liganded component. As long as binding material is present unliganded form will leave this region. The amount of binder ultimately trapped in the cortisol accumulation area will be dictated by several factors: The accumulated amount of cortisol, the strength of the interaction at the isoelectric point, the focusing time, etc. Therefore, on refocusing isolated components containing cortisol one should expect the steroid to be accumulated in the position of the liganded forms as borne out by experiment (Fig. 3, c and d). Since in desialylated plasma minor unliganded and major liganded components show identical isoelectric points, sequential transport is expected to occur. The finding that the actual cortisol elution position (pH 5.10) is lower than the position expected for cortisol in a gradient containing a uniform cortisol concentration (6 × 10^{-10} M). Prior to the activity measurement the cortisol excess was removed by dialysis.

**TABLE I**

Isoelectric positions of cortisol-binding components of asialotranscortin

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gradient without cortisol (unliganded components)</th>
<th>Gradient with cortisol (liganded components)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minor</td>
<td>Major</td>
</tr>
<tr>
<td>A*</td>
<td>5.38</td>
<td>5.58</td>
</tr>
<tr>
<td>B</td>
<td>5.38</td>
<td>5.57</td>
</tr>
<tr>
<td>C</td>
<td>5.36</td>
<td>5.56</td>
</tr>
<tr>
<td>D</td>
<td>5.36</td>
<td>5.56</td>
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</table>

* See Fig. 2.
Electrofocusing of Asialotranscortin

Fig. 3. Proof of the reversibility of the cortisol-controlled transition. Desialylated transcortin was fractionated as indicated in Fig. 2. Peak fractions were isolated, dialyzed against distilled water, and refocused separately under conditions opposite to those of the first separation. Conditions were as given in the legend to Fig. 2 except that the samples were introduced in one fraction of the sucrose gradient. In each case the application position was far to the right of the final positions. Refocusing of peak fractions isolated from a gradient without cortisol yielded binding Patterns a and b. Refocusing of peak fractions isolated from a gradient with cortisol yielded binding Patterns c and d. To the refocused protein samples 3.6 ng of [3H]cortisol were added. The location of radioactivity is indicated by the shaded areas. The isoelectric positions of the components observed in the first separation are indicated by the dots on the pH profiles.

Fig. 4. Evidence for cortisol accumulation due to interaction with cortisol-binding components. Steroid-free desialylated plasma (0.7 ml) was introduced at pH 4.68 (arrow) in one mixture of a previously formed pH gradient (pH 4 to 6) containing a unimodal pH profile (solid line). Cortisol-binding activity (—) was measured on 5-ml portions of 10-fold diluted fractions and radioactivity (— — —) on 0.2-ml aliquots of undiluted fractions. The ensemble of results is compatible with a release of protons linked to cortisol binding. In this respect the cortisol-asialotranscortin interaction resembles the well-documented oxygen-hemoglobin interaction (3–5). Since no polymeric material could be detected by gel filtration under the particular conditions of electrofocusing, it is unlikely that this cortisol-induced charge difference is related to a cortisol-controlled polymerization and depolymerization as reported for rat trans-
cortin (14). It is possible that the low ionic strength, inherent in electrofocusing, contributes to the peculiar behavior of asialotranscortin by changing its molecular architecture. The observation that ionic strength affects the cortisol-binding activity (Fig. 1) may lend support to this view.

Our experiments show that electrofocusing can profitably be used to study ligand-protein interactions. Furthermore, the results show that binding of nonionic substances, especially in cases of tight interaction (Fig. 3, d), may contribute to the microheterogeneity observed in many proteins. Finally as clearly indicated by the present results, detection of a radioligand, a method usually employed for following a macromolecular binding agent during separation, is not always an adequate technique.

Acknowledgments—We are indebted to Dr. W. Heyns and Dr. K. P. M. Heirwegh for stimulating discussions and for critically reviewing the manuscript.

REFERENCES

A Cortisol-induced Charge Difference in Desialylated Human Transcortin Detected by Isoelectric Focusing
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