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STEROID-PROTEIN INTERACTION AS STUDIED BY ISOELECTRIC FOCUSING*

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SUMMARY

Electrofocusing of sialic acid-free transcortin in the presence of trace amounts of [^{3}H] cortisol results in the complete separation between the steroid and the binding protein. This phenomenon can be explained by a charge alteration induced by cortisol binding.

THE METHOD described by Vesterberg and Svensson[1] under the name of isoelectric focusing in a natural pH gradient, results in the separation of proteins according to their isoelectric point. This procedure possesses a higher resolving power than most electrophoretic techniques and can theoretically be used to detect charge alterations on a protein caused by binding of non-ionic ligands provided the protein displays ligand-binding properties at its isoelectric point.

In view of a possible cortisol-induced charge difference, human transcortin was investigated by means of isoelectric focusing. Since native transcortin does not display measurable cortisol-binding properties at its isoelectric point (pI ≃ 4) use was made of sialic acid-free transcortin (asialotranscortin) which does exhibit this property (pI ≃ 5·5). When neuraminidase-treated human plasma, previously equilibrated with trace amounts of [1,2-^{3}H]cortisol, was submitted to isoelectric focusing at 4°C, the labeled cortisol was concentrated in a single peak at pH 5·07 and was found to be almost completely unbound as shown by simple equilibrium dialysis against distilled water. However, the cortisol-binding activity pattern, obtained subsequently by multiple equilibrium dialysis at pH 7·2, was characterized by the presence of a minor peak at pH 5·36 and a major peak at pH 5·56. A characteristic separation is shown in Fig. 1. From this and other electrofocusing experiments it could be concluded that the *association* of cortisol is accompanied by a *liberation of protons* whereas *dissociation* of the steroid-protein complex results in an *uptake of protons*. Such a mechanism entirely explains the unexpected behavior of traces of cortisol since it accounts for the existence of two isoelectrically different configurations (the liganded and the unliganded form) for the same protein molecule which will be separated by electrofocusing. Due to the reversibility of the cortisol binding and to the fact that unbound cortisol, as a result of its non-ionic nature, does not migrate in the electric field, the normal equilibrium between cortisol and asialotranscortin will always be shifted giving rise ultimately to cortisol-free binding protein and unbound cortisol. In successive steps involving the two varieties of asialotranscortin (pI 5·36 and 5·56), the entire trace load of cortisol will be accumulated at the anodic side of these two forms.

As far as the molecular basis of the cortisol-induced charge difference is concerned, two possible models may be considered. Cortisol could change directly the charge of asialotranscortin by inducing conformational changes which reduce the pK_a value of a certain side chain group. On the other hand it is possible

* A more detailed account of this study is given in the May (1972) edition of *J. Bioc. Chem.*

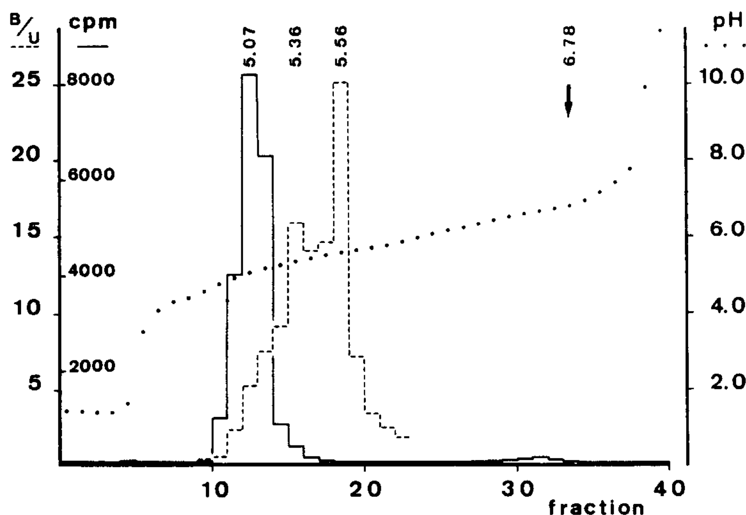

Fig. 1. Electrofocusing of the [^{3}H]cortisol-asialotranscortin complex in a preformed pH gradient. The sucrose gradient (from 46 to 4 per cent) contained 1 per cent fractionated ampholytes of a 5–7 pH range and was submitted to a final potential of 1000 V for 45 h at 4°C. The arrow indicates the pH of application of the steroid-protein complex. Fractions of 2·8 ml were collected and analyzed for pH (· · ·), radioactivity (——) and cortisol-binding activity (– – –), which was measured by means of multiple equilibrium dialysis at 4°C and pH 7·2.

that binding itself does not change the charge of the protein but upsets the equilibrium between an inactive and a more negatively charged active form of the binding protein. These isoelectrically different configurations may arise from the fact that the base form of a certain side chain group is essential for maintaining the structural integrity of the binding site. Since the release of protons as a result of cortisol binding is observed around pH 5·5, the cortisol-linked group may be a carboxyl as well as an imidazole group.

REFERENCE

1. Vesterberg and H. Svensson: *Acta. Chem. Scand.* **20** (1966) 820.

DISCUSSION

Rosner: There are discrepancies in some of our results; I don't know quite how to explain them—perhaps you can. Rather than work with plasma or diluted plasma, we used large amounts of purified transcortin (~ 5 mg) so that we could examine the proteins eluted from columns. The columns were run at low temperatures and so that we could see binding we added a lot of cortisol counts. This is still a trace amount in relation to the large amount of transcortin used (this CBG has about 80% of its binding sites occupied by cortisol), and interestingly enough, we see not asialoprotein, but intact CBG as a single peak of protein and a single peak of cortisol, at pH 3·94. I don't know where that discrepancy comes from.

Van Baelen: Have you localized the protein after the separation by means of another technique than following the radioligand?

Rosner: We have examined highly purified CBG by isoelectric focusing. After adding tritiated cortisol to about 5 mg of CBG we found that both the steroid and

the protein focused sharply at pH 3·94. However when we tried to refocus the CBG after reisolating from the focusing column it would no longer bind and the protein was spread over many pH units.

Van Baelen: Well, we performed gel filtration of these protein peaks, and the binding activity pattern was always eluted together with serum albumin, suggesting that the Stokes radius is not changed during the separation, or by treatment with neuraminidase.

Rodbard: We have also used isoelectric focusing to study steroid-protein binding, modified by use of polyacrylamide gel as a medium rather than a sucrose gradient. This enables us to apply isoelectric focusing on an analytical level. (Doerr P. and Chrambach A.: *Anal. Biochem.* **42** (1971) 96). This technique was able to demonstrate heterogeneity of antibodies to estradiol, and offers a potential method for purification of antibodies having improved specificity for either oestradiol or oestrone.