STUDIES OF HUMAN TRANSCORTIN AT DIFFERENT pHs: CIRCULAR DICHROISM, POLYMERISATION AND BINDING AFFINITY

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1. Introduction

Human transcortin or corticosteroid binding globulin (CBG), is a glycoprotein which specifically binds corticosteroid hormones in the blood [1]. It can now be obtained in a pure and active state by affinity chromatography [2,3]. The physical parameters, the amino acid and the osidic composition and the N-terminal sequence have been given in a previous publication [4].

We present in this paper, a circular dichroism study of native and heat-inactivated transcortin. The dichroic spectra variations of native transcortin with pH are shown in correlation with its binding affinity and its polymerisation capacity.

2. Materials and methods

All reagents were Analytical grade and solutions were made with deionized water which have been glass distilled. Cortisol was purchased from steraloids and used without further purification. Transcortin was prepared by affinity chromatography as previously described [3]. The concentration of cortisol in the transcortin–cortisol complex was always taken as one mole of cortisol to one mole of transcortin [5] using a mol. wt. of 49 500 for transcortin [4]. The experiments were carried out at room temperature (23°C). At this temperature the association constant is $0.52 \times 10^8\ M^{-1}$ [6]. Inactivated transcortin was obtained by incubating a solution of 10 mg of transcortin/1 ml of distillated water at 60°C for 30 min. Indeed, De Moor et al. [7] have shown that human transcortin was irreversibly inactivated at this temperature.

To attempt reversing the heat inactivation of transcortin, 10 mg of the inactivated mixture transcortin–cortisol (molar ratio, 1:1) were dissolved in 1 ml of Tris 0.05 M-HCl buffer pH 8.0, to have a 0.2 mM concentration; 18 µl of a 10 mg/ml cortisol ethanolic solution and 3.1 mg of dithiothreitol (Calbiochem) in powder were added to the solution for obtaining respective concentrations of 0.5 mM and 20 mM. The solution was kept 2 h in the dark at 23°C before testing for binding affinity.

For testing variations of binding capacity with pH, 5 mg of native transcortin–cortisol complex (molar ratio, 1:1) were dissolved in 1 ml of sodium phosphate 0.05 M, pH 7.4 buffer; then 1 µCi of [1,2-^{3}H]cortisol (spec. act.: 30 Ci/mM, New England Nuclear) was added to the solution. The mixture was incubated 90 min at 37°C to reach an equilibrium between transcortin and [^{3}H]cortisol. It was then divided into five aliquots of 0.2 ml which were adjusted to the desired pH with NaOH 1 M or HCl 1 M; the pH was measured with pH strips (Merck) of high sensibility (0.2 pH units). 15 µl of each solution were used for polyacrylamide gel electrophoresis and 100 µl were diluted to 1 ml with sodium phosphate 0.05 M buffer at the same pH. To test the binding affinity, the solutions were cooled for 10 min at 0°C in melting ice. They were then added up with 0.5 ml

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Abbreviations: CBG corticosteroid binding globulin; CD, circular dichroism.

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of dextran-coated charcoal suspension at the same pH and temperature (10 mg/ml of charcoal Norit Prolabo and 1 mg/ml of dextran T70 Pharmacia). The suspensions were kept at 0°C for 30 min and centrifuged at 2°C. 0.5 ml of the clear solutions was counted with a dioxane scintillator in a three channels scintillation spectrometer (Intertechnique, SL 30 type coupled to a multi 8 system).

Polyacrylamide gel electrophoreses were performed with 7.5% acrylamide and Tris-glycine, pH 8.3, buffer according to the method of Davis [8] in a 'disc electrophoresis' Canalco device (1200 model). Gels were coloured with Coomassie brillant blue R (Gurr) according to the method of Vesterberg [9].

Circular dichroism (CD) measurements were carried out using a Jobin Yvon R. J. Mark III dichrograph. The temperature was maintained at 23°C and the spectra recorded from 180 to 400 nm. The molar ellipticity [Θ] was calculated on the basis of the concentration in amino acid residues for the spectra corresponding to the amide chromophore, that is between 180 and 240 nm. The mean residue weight, determinated from the composition of the protein moiety, was 115 [4]. Owing to the weak concentration of *N*-acetyl glucosamine in transcortin the contribution of the carbohydrate moiety to the dichroic spectra was neglected. The shape of the experimental dichroic spectra obtained were converted into gaussian curves using a Du Pont Curve resolver. It yielded negative maxima at 205, 214 and 222 nm. The α-helix content then was estimated from the $n \rightarrow \pi^*$ transition, i.e. the band centered at 222 nm, taking the value of −30620 for the helix as a standard. The amount of β structure was estimated in a similar manner using the band at 214 nm and taking −7040 for the β-form as a standard. The values of the standards were determined for the dichrograph used in a 180–250 nm range of wavelengths, from six proteins [10] from the procedure of Chen et al. [11].

For the remaining parts of the spectra, between 240 and 400 nm, the molar ellipticity was calculated from an assumed mol. wt. of 36 135. This mol. wt. corresponds only to the protein moiety, taking into account the fact that the whole glycoprotein has a mol. wt. of 49 500 and contains 27% of glycans.

In general, CD measurements were carried out in 0.214 M NaF at pH 7.1. When the pH varied, concentrated perchloric acid was added to the solution with a micropipette and the pH measured with a Radiometer pH meter.

The CD spectra of the free cortisol was carried out in a solution in an ethanol–water mixture.

3. Results and discussion

3.1. *Temperature inactivation of transcortin*

Incubation at 60°C for 30 min of a 0.2 mM solution of human transcortin–cortisol complex (molar ratio, 1:1) in distilled water or in Tris 0.05 M HCl, pH 8.0, buffer results in a complete and irreversible inactivation. It was been demonstrated, [4], that a partial polymerisation occurred concomitantly by aggregation and disulfide bridge dimer formation and that an equilibrium was probably reached in which 8 monomers give rise to 1 dimer with two disulfide bridges and 6 monomers with free thiol groups.

Addition of 20 mM dithiothreitol to cleave the disulfide bridges and a 2.5 M excess of cortisol to protect the binding site does not reverse the inactivation process.

3.2. *Influence of pH on the stability of pure transcortin*

Stability of transcortin in solution at different pHs (0.8, 7.4, 6.0, 5.0 and 4.0) has been tested by two different means: polyacrylamide gel electrophoresis and binding affinity assay. Tests were performed immediately after reaching the desired pH.

A very minor zone, which is difficult to see on fig.1, appears progressively above the monomer zone when the pH decreases from 8.0 to 5.0. At pH 4.0, that zone increases suddenly and two or three more zones appear above (fig.1). The aspect at pH 4.0 is similar to heat- or urea-inactivated transcortin and, as mentioned previously, is due to polymerisation.

For testing the binding affinities, each solution which contains an equal concentration of the transcortin–[^{3}H]cortisol complex was treated by dextran-coated charcoal at 0°C to adsorb free cortisol. This low temperature was chosen to provide the maximum affinity for transcortin [12]. The radioactivity remaining after centrifugation reflected only bound cortisol. The results in table 1 show that maximum binding capacity is obtained at pH 8.0, and that capacity decreases when the pH decreases. If we measure the percentage inhibition with respect to the

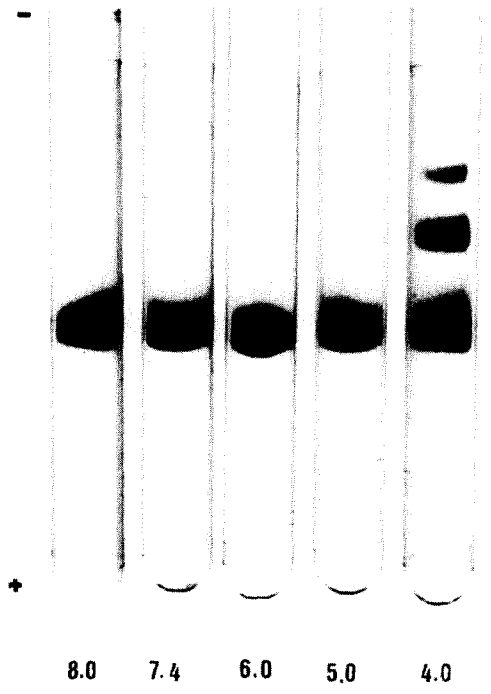

Fig.1. Polyacrylamide gel electrophoresis of transcortin (75 μg) in solution at different pHs. Electrophoresis was conducted from the top to the bottom in 7.5% acrylamide pH 8.3 buffer at 4 mA per tube during 75 min according to the method of Davis [8].

binding capacity at pH 8.0, it can be seen that 93% of the binding power is lost at pH 4.0. This result is similar to the appearance of heat-inactivated transcortin as shown from polyacrylamide gel electrophoresis. It should be noted that pH 4.0 is the isoelectric point of the transcortin–[^{3}H]cortisol complex determined from isoelectric focusing of human serum [13].

These binding affinities determined on pure human transcortin corroborate the results obtained on whole serum [7,14] but the polymerization at low pH has never been observed as impure human transcortin does not polymerise [4].

Table 1
Variations of transcortin binding capacity with pH

pH	Bound cortisol c.p.m.	Inhibition percentage
8.0	12.773	0
7.4	11.563	9
6.0	11.543	10
5.0	8.881	31
4.0	909	93

1 ml of transcortin–cortisol complex solution $1 \cdot 10^{-5}$ M (0.5 mg/ml) in sodium phosphate 0.05 M buffer containing 0.2 μCi of [1,2^{3}H]cortisol is treated by 0.5 ml of dextran-coated charcoal suspension at 0°C during 30 min. After centrifugation at 4°C, 0.5 ml is counted.

4. Circular dichroism study

Fig.2 shows the u.v. spectrum of cortisol in an ethanol–water solution. Fig.2 also shows the corresponding dichroic spectra where it it possible to

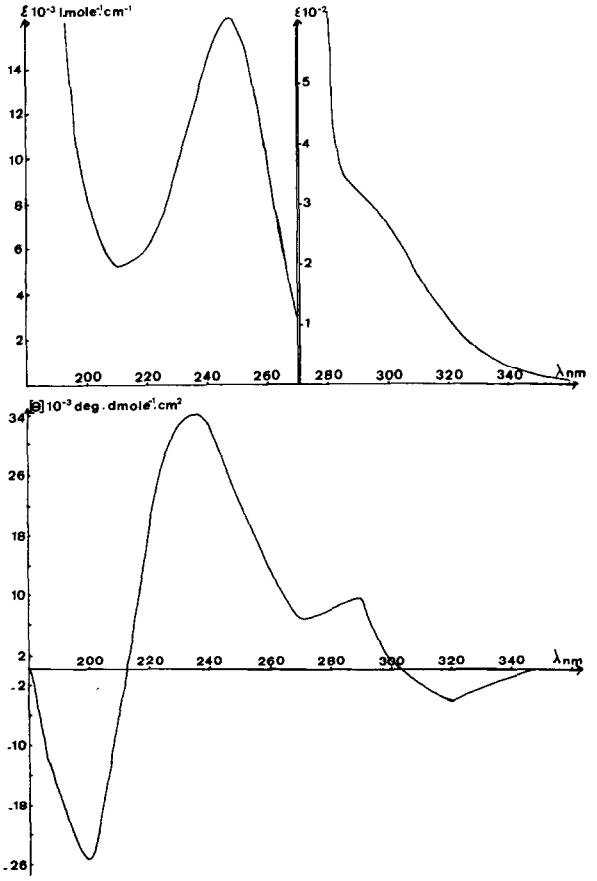

Fig.2. U.v. (upper part) and dichroic (low part) spectra of cortisol in ethanol–water mixture.

see four dichroic bands at 320, 290, 236 and 200 nm. These correspond to ellipticities of 4×10^3; 9.5×10^3; 33.5×10^3 and 25×10^3 deg·dmole^{-1}·cm^2 respectively. Hence, the dichroic spectrum of the cortisol overlaps the dichroic spectra of the amide chromophore in the 180–240 nm range. The molar ellipticities are comparable to the cortisol and the amide chromophore but owing to the fact that the ratio of cortisol/transcortin, in the cortisol–transcortin complex is 1/1, for one mole of cortisol, it corresponds to 295 residues of amino acid. Therefore, the contribution of the cortisol can be neglected in the CD spectra corresponding to the amide chromophore.

We have studied the native CBG as the CBG–cortisol complex and the denaturated CBG without cortisol at pH 7.9 and 7.1 in 0.214 M NaF aqueous solution at this short wavelength region. The corresponding dichroic spectra are shown in fig.3. There is a difference between the secondary structure of each transcortin. One finds 35% and 22% of α-helix for native and denaturated CBG respectively. This indicates that the binding of the cortisol has an effect on the conformation of the protein backbone.

Since it is well-known [7] that the binding affinity of serum decreases with the pH and becomes zero when the pH = 2, we have recorded the dichroic spectra of the CBG–cortisol complex between pH 7.9 and 4. Below pH 4, CBG precipitates. The results of such a study are shown in fig.4. The analysis of the dichroic spectra in term of α-helix and β-sheet structures are given in table 2. One observes that at the three pH values of 7.9, 7.2 and 6.5, the percentage of β-conformation is zero. This percentage rapidly increases when pH decreases further. Indeed at pH = 5.9 this percentage is 31%. Such an increase corresponds to an organization of the protein moiety, as under the same conditions of decreasing pH, the percentage of the α-helical form only decreases by 5%. At the lowest

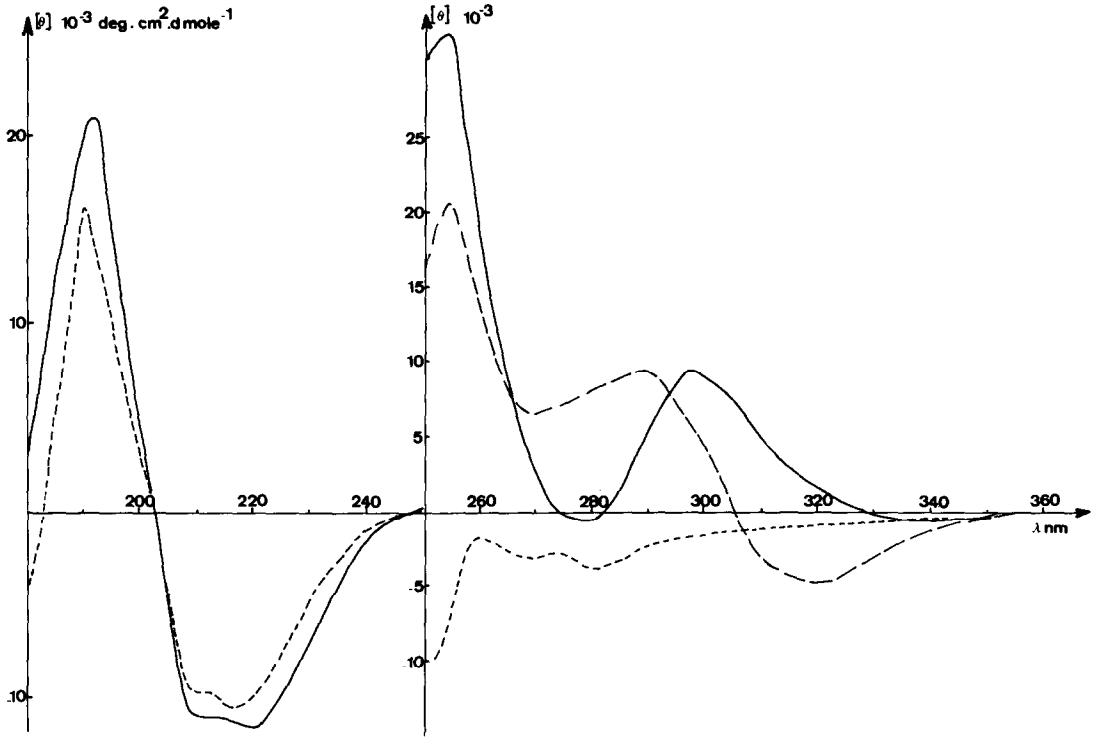

Fig.3. Dichroic spectra of CBG in different conditions: (————) native CBG; (- - - - - -) Denatured CBG without cortisol; (– – – –) Denaturated CBG with cortisol. The ellipticities expressed in degree·cm^2·decimole^{-1}, are calculated taking as mean residue weight M = 115 for the dichroic spectra corresponding to the amide chromophore (between 180 and 250 nm) and M = 36 135 for the dichroic spectra corresponding to extrinsic chromophore (between 250 and 360 nm).

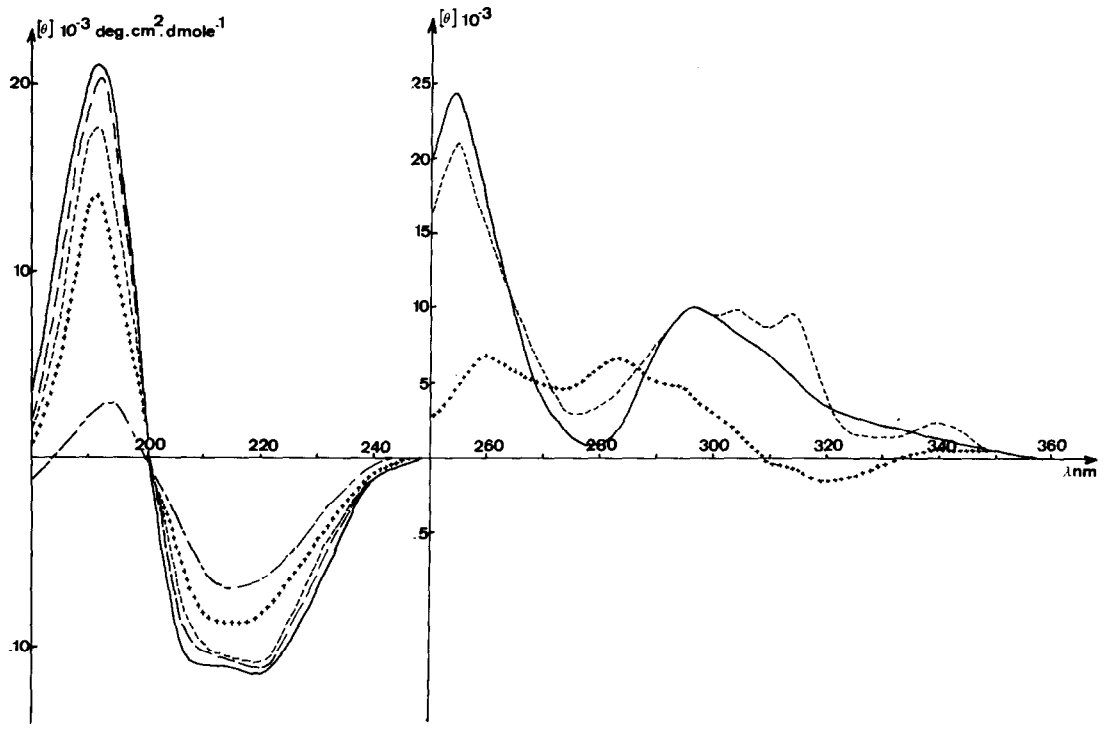

Fig.4. Dichroic spectra of native CBG at different pHs (————) pH 7.9, 7.2 and 6.5; (– – – –) pH 5.9; (- - - - - - - -) pH 5; (+ + + +) pH 4.5; (— - —) pH 4. [θ] are expressed as in fig.3.

pH values obtainable, i.e. from 5.9 down to 4.5, the percentage of β-structure remains approximately constant but the percentage of α-helical form decreases slightly.

Now let us consider the dichroic spectra recorded between 240 and 400 nm, i.e. corresponding to the native CBG and the denatured CBG, with and without cortisol. In this region the dichroic bands have two origins. These arise from the aromatic residue side chains of the protein moiety and the cortisol. Of course the spectrum corresponding to the denaturated CBG without cortisol is only due to the aromatic residue side chains. Since it is well-known that a Cotton effect can be induced in a chromophore drug or a dye bound to proteins, the dichroic spectrum corresponding to the cortisol bound to the CBG could be different from the CD spectrum of the free cortisol. Moreoever interactions can take place between cortisol and aromatic side chains, probably with a tryptophan residue, where such interactions can give considerable changes in the CD spectra corresponding to these side chains. Unfortunately, the assignement of individual electronic transition, which might be required for a good interpretation, is not obvious in this case. The CD spectrum of the denaturated CBG with cortisol minus the CD spectrum of the denaturated CBG without cortisol corresponds, of course, to the CD spectrum of the free cortisol in 0.214 M NaF aqueous

Table 2
Secondary structure of transcortin at different pHs

pH	Percent helical structure	Percent β-structure
7.9, 7.2, 6.5	36	0
5.9	31	31
5	28	32
4.5	18	35

solution. It is interesting to note that the positions of the peaks and the magnitudes of the dichroic bands are the same as those of cortisol in ethanol–water solution. On the contrary, the dichroic spectrum of the CBG–cortisol complex presents two maxima which do not correspond to the position of maxima observed with free cortisol, and the negative band at 320 nm is not observed as seen in fig.3. In this case, it is not possible to subtract the contribution of the aromatic residue side chains, as it is impossible to obtain CBG in the native state and without cortisol. It is interesting to observe that the shape of the spectrum is reminiscent of the spectrum of the free cortisol but with some modification. The peak situated at 297 nm for native CBG could correspond to the peak observed at 290 nm with free cortisol. The shift would be due to an electronic modification arising from the binding of the cortisol. Moreover, in the tail of this dichroic band a shoulder is observed at 305 nm. At this wavelength no electronic transition of the cortisol exists; therefore, this shoulder could be due to a tryptophan which is implicated in the binding with cortisol, since the dichroic bands due to the side chain of tryptophan are generally observed in this domain.

It is difficult to give a precise assignement to the band situated at 260 nm. At this wavelength and given the concentrations used to record the dichroic spectra in this domain, the contribution of the amide chromophore cannot be neglected. However, it is interesting to observe that the intensity of this band, which is in proximity to the 248 nm cortisol band, decreases from the native to the denaturated CBG.

The dichroic spectra at different pHs were recorded and are shown in fig.4. At pH 7.9, 7.2 and 6.5, the spectra are superimposable and correspond to the native CBG, that is when the cortisol is bound to the glycoprotein. In this case the spectrum is reminiscent of the one observed for native CBG (fig.3): the positions of the extrema are exactly the same and the magnitudes of the dichroic bands are in good agreement with each other. When pH decreases, the shape of the spectrum is varying. More especially, at pH 5 new bands appear. The positive bands at 305 and 315 nm could be due to the tryptophan transitions and the one at 325 nm to the cortisol which presents a negative band in this region. At the lowest pH, (4.5) the dichroic spectrum is reminiscent of the spectrum obtained with cortisol denaturated CBG but does not precisely represent the sum of denaturated CBG and of free cortisol dichroic spectra. This is because at pH 4.5 the denaturation of CBG is incomplete.

5. Conclusion

The transcortin we have used in this work is extremely pure. This was shown by the polymerisation observed at pH 4. This polymerisation is never observed with an impure form of transcortin [4]. Moreover, since it is known that the presence of cortisol in the binding site is an essential condition to the activity of purified transcortin [5], it appears that a correlation between the secondary structure and the biological activity of the transcortin exists. The results we have obtained are summarized below:

(1) The inhibition of the transcortin binding capacity essentially takes place between pH 5 and 4.

(2) A reorganisation of the structure of the protein moiety is observed between pH 6.5 and 5.9.

(3) A decrease of the helicity ratio is observed between pH 5 and 4. It appears therefore that, in the limits of experimental accuracy of CD measurements to determine the amount of β-structure, no appreciable change of binding activity is taking place after the appearance of a large percentage of β-structure between pH 6.5 and 6.

On the other hand, the sudden decrease of protein activity at low pH is likely to be correlated with the disappearance of a well-defined helical region.

Other biochemical and physical experiments would be of course necessary, in order to precise this first observation of a structure–function relationship in transcortin.

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