APPLICATION OF INDUCED OPTICAL ACTIVITY TO THE STUDY OF PROTEIN DENATURATION

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1. Introduction

Optical rotatory dispersion and circular dichroism are widely used as a means of studying conformation changes in biological macromolecules under various environmental conditions [1].

Most denaturation processes are investigated by these techniques since the different conformations of biopolymers (α helix, β form or randomly coiled) are well discerned by their optical properties [2].

This report presents another approach for investigation protein denaturation which involves measurement of the induced optical activity exhibited by a non-covalently bound substrate when human serum albumin (HSA) is treated with urea and 2-chloroethanol. This study was also undertaken to gain further insight into the extrinsic Cotton effect mechanism.

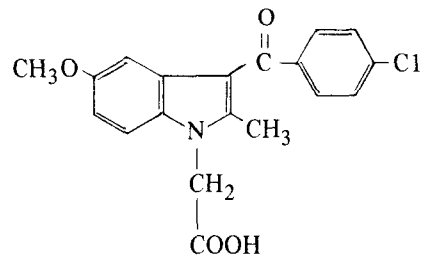
HSA was selected because its denaturation has been extensively studied [3–6] and is has the ability to induce asymmetry in a great number of bound molecules which are optically inactive when unbound.

The extrinsic Cotton effect was quantitatively measured using an equilibrium dialysis technique to evaluate the binding parameters (number of binding sites (n) an association constants (K)) of the complex.

2. Materials and methods

HSA (electrophoretic purity 99%) was obtained from Centre National de Transfusion Sanguine and was used without further purification.

The substrate which has been used in all experiments is 2-methyl-3-parachlorobenzoyl-5-methoxy indolyl-1 acetic acid.



Solutions were made up in 0.1 M sodium phosphate buffer pH 7.4. Equilibrium dialysis experiments were carried out using Visking Tubing 20/32 inch.

CD measurements were performed on a Roussel–Jouan Model II dichrograph (cell path length 5 cm over the range 300–400 nm) and absorption spectra were recorded on a Cary 14 spectrophotometer. For equilibrium dialysis, the tubing was soaked in buffer over-night and rinsed three times in distilled water prior to use.

The volume in the bag was 10 ml and 20 ml outside. All the experiments were carried out at 25°C.

HSA concentration inside the bag was 510^{-5} M, based on a molecular weight of 69 000.

Dialysis ran for 16 hr, a time which was previously shown to be sufficient for complete equilibration.

Substrate concentrations were determined in the outside solution by UV spectrophotometry (λ = 350 nm, ϵ = 10 000).

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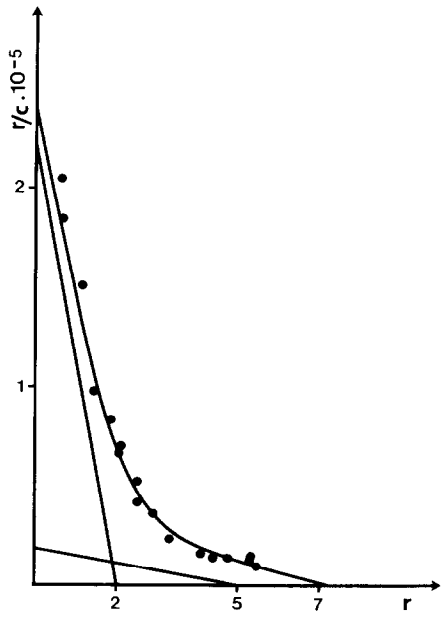

Fig. 1. Scatchard representations of the binding of the substrate to HSA in phosphate buffer 0.1 M, pH 7.4: r = molar ratio of bound substrate to protein; c = concentration of unbound substrate.

The inside solution was directly analyzed by CD technique.

The binding parameters were determined according to Scatchard [7] and Rosenthal [8].

3. Results and discussion

3.1. *Binding parameters and optical properties of the substrate–HSA complex*

As expected from its molecular structure, the substrate selected for this study does not show any optical activity over the range 200–400 nm when free in solution or in presence of fully denaturated HSA (urea 8 M)

Its UV spectrum exhibits a great number of electronic transitions which become optically active upon binding to HSA (for a general review of this phenomenon see Chignell [9]). The presence of the CD spectrum of HSA makes the investigation of the region below 300 nm difficult, particularly when one is dealing with denaturation processes.

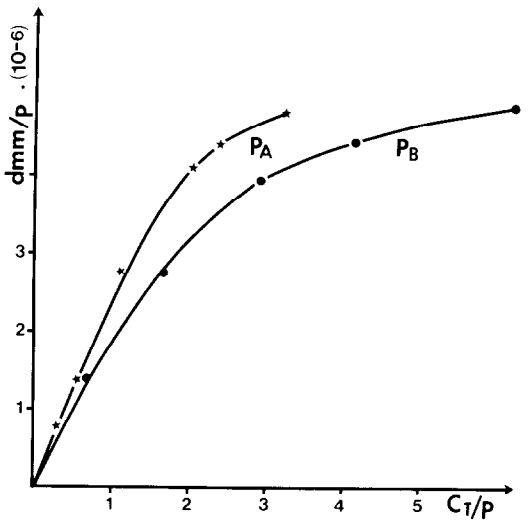

Fig. 2. Variations of the induced optical activity (λ = 355 nm) of the substrate over HSA concentrations, P, as a function of its total concentration C_T over P for two protein concentrations P_A = 14.5 μm, P_B = 50 μm.

Therefore, the only electronic transition taken into account for quantitative CD measurement was located at 350 nm.

Before examining the influence of urea and 2-chloroethanol on the induced activity of the ligand, the binding parameters of the complex were determined by equilibrium dialysis experiments.

It is apparent, from the Scatchard representation of the experimental data (fig. 1), that HSA exhibits more than one type of binding site for the substrate.

Assuming two different kinds of binding sites, the experimental curve can be decomposed, according to Rosenthal [8], into two straight lines which correspond to specific binding ($n_1 = 2, K_1 = 1.1 \times 10^5$ M^{-1}) and non-specific binding ($n_2 = 5, K_2 = 3.6 \times 10^3$ M^{-3}).

The binding of the selected substrate to HSA has also been investigated by circular dichroism, using the method of Halfman and Nishida [10]. This method, which has been applied successfully in fluorescence spectroscopy, is valid for DC if one assumes the author's general hypothesis, namely that the binding parameters and $\Delta\epsilon$ of the bound ligand are independent of protein concentration.

The titration curves, $d_{mm}/P = f(C_T/P)$ where d_{mm}

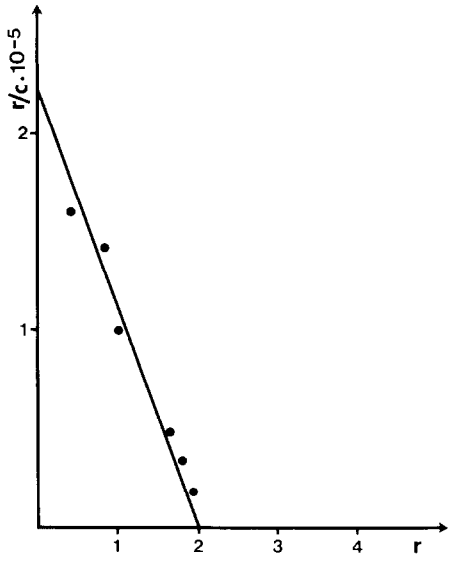

Fig. 3. Comparison between the Scatchard representation of the substrate–HSA complex obtained by CD (•) and by equilibrium dialysis experiments (solid line).

is the induced CD amplitude, C_T the total substrate concentration (bound plus unbound) and P the protein concentration are represented fig. 2 for two protein concentrations P_A and P_B.

Following Halfman and Nishida [10]

$$r = \frac{\left(\frac{C_T}{P}\right)_A - \frac{P_B}{P_A}\left(\frac{C_T}{P}\right)_B}{1 - \frac{P_B}{P_A}}$$

$$c = \frac{P_A P_B}{P_A - P_B}\left[\left(\frac{C_T}{P}\right)_B - \left(\frac{C_T}{P}\right)_A\right]$$

$(C_T/P)_A$ and $(C_T/P)_B$ are the molar ratios of the total substrate to HSA concentrations, P_A and P_B respectively which correspond to the constant ordinate value.

The different values of r and c obtained by this method can be plotted according to Scatchard. The relationship between r/c and r is linear and is identical to what has been obtained by equilibrium dialysis experiments (fig. 3).

This result confirms the fact that, at least for albumin, the ability to induce asymmetry in a ligand is very dependent upon the nature of the binding sites.

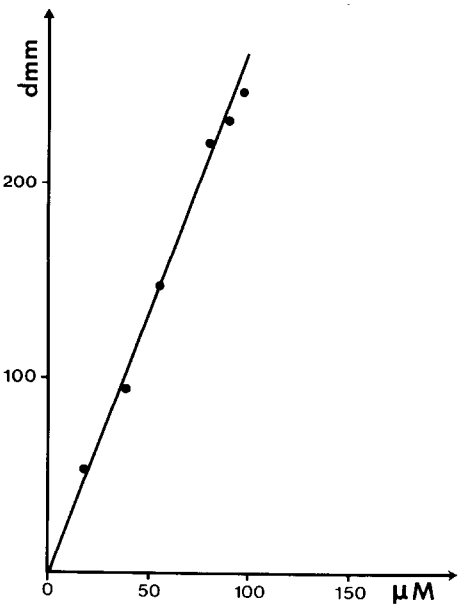

Fig. 4. Variations of the induced CD amplitude (λ = 355 nm) of the substrate as a function of the concentration of bound substrate to specific sites [HSA] = 50 μm.

The extrinsic Cotton effect is only generated by the high affinity sites. Such a conclusion has been reached with Sulfaethidole–BSA [11] and Dicoumarol–HSA [12] complexes.

As indicated in fig. 4, the variations of the induced CD amplitude as a function of the concentration of bound molecules to specific sites are linear. The slope of this line gives the value of the induced $\Delta\epsilon$, equal to 5.7.

3.2. *Influence of urea and 2-chloroethanol on the optical properties of the substrate–HSA complex*

Although the interaction between denaturing agents and substrate protein or polynucleotide complexes has been studied in some detail by optical activity measurements [13–17], little is known concerning the variations of the induced optical activity during denaturation processes as a function of the binding property of a non-covalently bound ligand.

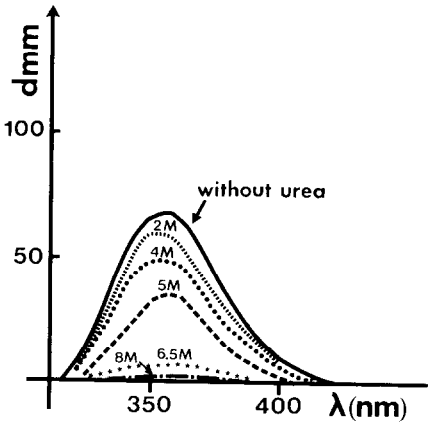

Fig. 5. Induced CD spectrum of the substrate over the range 300–400 nm for various urea concentrations. [HSA] = 16 μm.

3.2.1. *Influence of urea*

Urea is known to alter to a great extent the tertiary structure of albumin [4]. The transition α helix → random coil of this protein has been studied by circular dichroism. This method has shown a large decrease of the helix content of HSA during titration with urea [3]. The extrinsic Cotton effect exhibited by the ligand is also markedly affected by denaturation processes. The induced circular dichroism spectrum of the substrate were recorded at increasing concentrations of urea (fig. 5).

Klotz and Shikama [18] have reported that the ability of BSA to bind small molecules is greatly altered in presence of urea. Our results raise the following question concerning the influence of this denaturing agent on the induced $\Delta\epsilon$: are the observed variations due to a regular decrease of the amount of bound substrate (in this case, the induced $\Delta\epsilon$ should be constant) or to a modification in the properties of the binding sites to induce asymmetry in the ligand?

To answer this question, equilibrium dialysis experiments have been performed in presence of 5 M urea. Under these conditions, HSA is not fully denaturated and can still exhibit some binding properties, as indicated in fig. 6. The decomposition of the experimental curve is much more difficult to carry out in presence of 5 M urea than with the native protein.

In order to fit the experimental data, the binding on the more specific sites must be represented by a slightly curved line which suggests that there exists more than one species of binding sites.

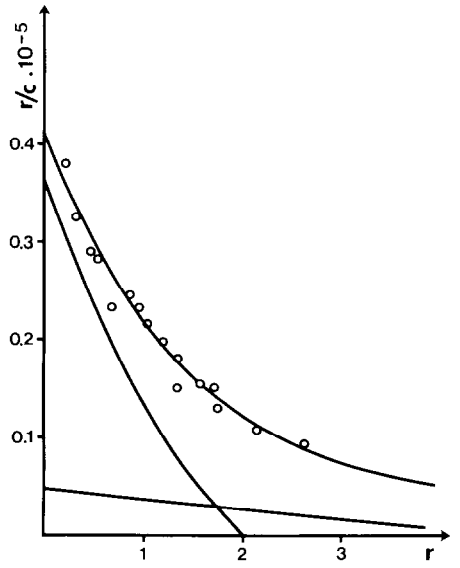

Fig. 6. Scatchard plots of the binding of the substrate to HSA in presence of urea 5 M in phosphate buffer 0.1 M, pH 7.4.

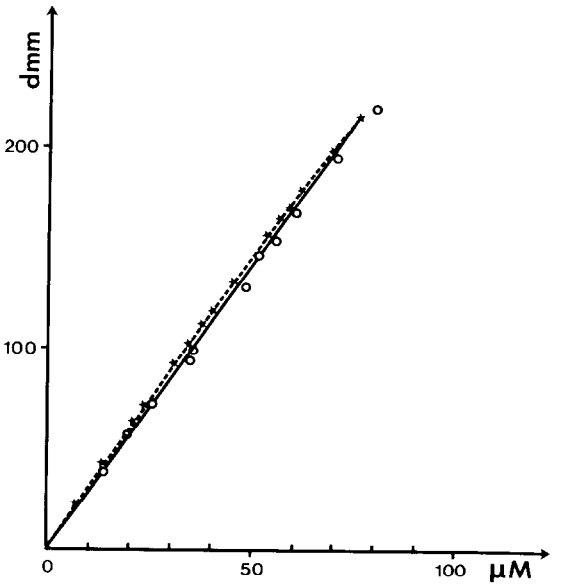

Fig. 7. Variations of the indiced CD amplitude at 355 nm of the substrate as a function of the concentration of bound substrate to specific sites. (x–x–x) with 5 M urea; (o–o–o) without urea; [HSA] = 16 μm.

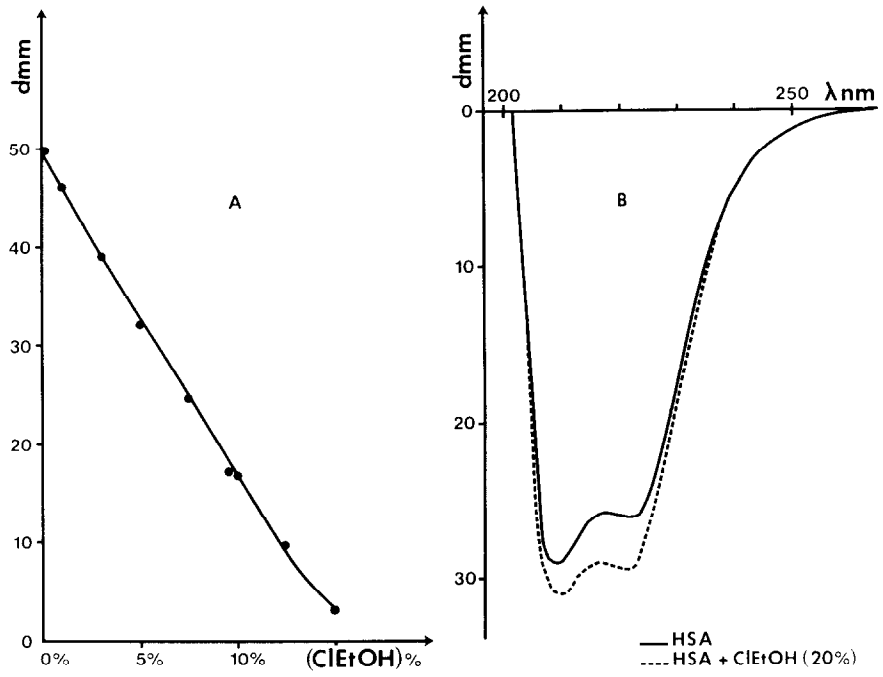

Fig. 8. A – Variations of the induced CD amplitude of the bound ligand as a function of increasing concentration of 2-chloroethanol. [HSA] = 14.5 μM; [substrate] = 31 μM. B – Variations of the CD spectrum of HSA upon addition of 2-chloroethanol. [HSA] = 14.5 μM.

During the same experiment, the inside solution of the dialysis bag was analyzed by the CD technique. Assuming that the observed extrinsic Cotton effect was due only to molecules bound to sites of higher affinity, the variation of the CD amplitude of the ligand as a function of bound substrate (fig. 7) has been found to be nearly identical with or without urea. This result suggests that, in contrast to the binding parameters, the value of the induced $\Delta\epsilon$ is not modified upon mild denaturation conditions.

We have attempted to confirm the above assumptions by investigating the influence of another denaturing agent : 2-chloroethanol.

3.2.2. *Influence of 2-chloroethanol*

2-Chloroethanol, like dioxane or aliphatic alcohols, tends to increase the ordered structure of native proteins [3–9].

The CD variations of HSA and the variations of the induced CD amplitude of the bound substrate at constant wavelength (λ = 355 nm) are represented in fig. 8. It should be noted that the optical properties of the bound substrate are much more sensitive to this denaturating agent than HSA, which has a high α helical content (60–70%) in the native form.

As for urea, the binding parameters are greatly affected. Within the limits of experimental errors, the Scatchard representation of the equilibrium dialysis experiment performed in presence of 2-chloroethanol, is indicative of one species of binding sites (fig. 9).

In this case, the variations of the induced optical activity should be linear with the concentration of bound substrate, as found previously (see fig. 4).

The titration curve represented in fig. 10 shows in fact that the conclusion drawn from the dialysis experiment is incorrect since these variations are not linear regardless of 2-chloroethanol concentration.

From these preceeding observations, one cannot deduce any value for the induced $\Delta\epsilon$.

To overcome this difficulty, we have assumed that the induced $\Delta\epsilon$ remains constant ($\Delta\epsilon$ = 5.7) when the protein is partly denaturated and we have tested

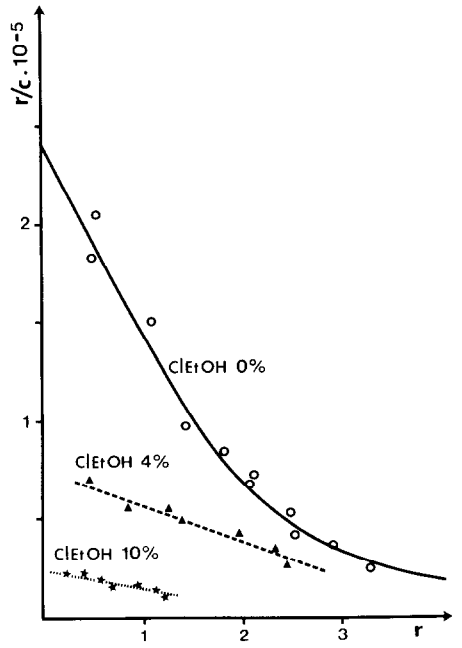

Fig. 9. Scatchard representation of the binding of the substrate to HSA in presence of various concentrations of 2-chloroethanol.

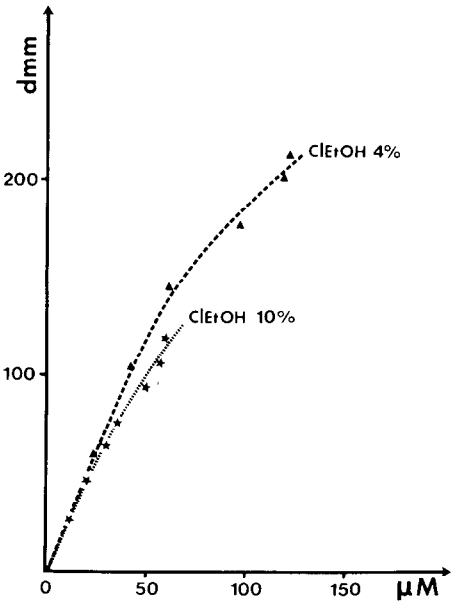

Fig. 10. Variations of the induced optical activity of the substrate at constant wavelength (λ = 355 nm) as a function of the bound substrate concentration for two 2-chloroethanol concentration. ▲–▲–▲ [2-chloroethanol] = 4%; x–x–x [2-chloroethanol] = 10%.

the validity of this hypothesis as follows: When the equilibrium dialysis is performed in presence of 4% 2-chloroethanol, the analysis by CD of the inside solution of the bag allows the determination of the concentration of bound substrate to the specific site (B_s), assuming a constant values of the induced $\Delta\epsilon$. Furthermore, the total concentration of bound and unbound substrate ($B_s + B_{ns}$) is known from dialysis experiments. Therefore, the extent of binding of the substrate can be determined from these two values (fig. 11).

The similarity between the experimental data (o) and the curves which have been constructed is satisfactory enough to conclude that the hypotheses is valid: there is no modification of the induced $\Delta\epsilon$ when HSA is partly denaturated by 2-chloroethanol.

It should be pointed out that the shape of the titration-curve observed in CD may provide a new means of observing the presence of non-homogeneous binding sites which would be barely detectable by equilibrium dialysis experiments.

The investigations of the dependence of the induced

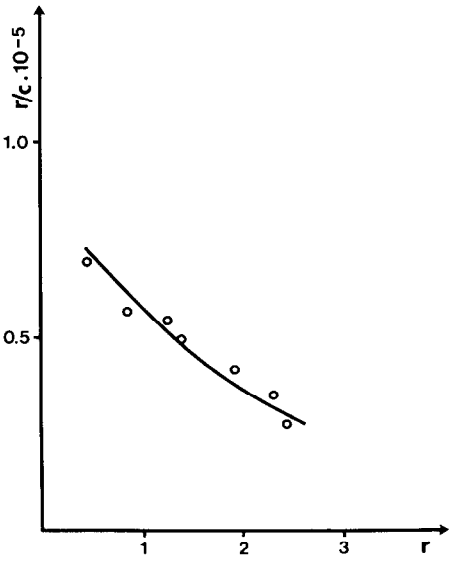

Fig. 11. Comparison between the Scatchard representation deduced from equilibrium dialysis (points) and CD experiment (solid line) assuming a constant induced $\Delta\epsilon$ of 5.4.

CD signal exhibited by a bound substrate on the concentration of denaturing agents, such as urea and 2-chloroethanol, strongly suggest that the observed variations are proportional to the amount of bound substrate.

The induced $\Delta\epsilon$ remains constant in the range of our experimental conditions, which has included only mild denaturation conditions. Whatever, the mechanism of optical induction is, this finding means that the geometric interaction between the chromophore of the substrate and the amino acid residue which generates the extrinsic Cotton effect does not vary. It is also important to note that no correlation appears between the induced $\Delta\epsilon$ and the binding parameters of the complex.

A variety of non-covalent interactions are involved in the binding of a small molecule to HSA, such as hydrogen bound, electrostatic and hydrophobic interactions, charge transfer ... A simple explanation of our CD investigation is to consider that, as soon as the active centers of the native protein are slightly disturbed by urea, they become absolutely unable to bind substrate molecules. Consequently, for a given urea concentration there would be an equilibrium between native and disturbed centers. It can be easily shown that this assumption leads to a constant induced $\Delta\epsilon$ and an apparent association constant for the specific sites smaller than that of the native protein. However, it seems unlikely that such a situation exists because urea is believed to alter progressively the geometry of the protein as well as its binding sites. In these conditions our results suggest that the interaction between binding sites and substrate can be classefied in two groups: the first one, which includes interactions responsible for the observed induced optical activity, is not affected by denaturing agent, while under the same condition the second is greatly altered.

Further work is in progress to elucidate this point.

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