CIRCULAR DICHROISM STUDIES ON THE BINDING OF L-TRYPTOPHAN TO HUMAN SERUM ALBUMIN

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1. Introduction

The binding of L-tryptophan to plasma proteins is an important phase in its general metabolism *in vivo* and has been the subject of several papers [1–6]. Although equilibrium dialysis or gel filtration was used in these studies, circular dichroism (CD) measurements, however, seem to be an attractive alternative method, giving faster and more reliable results in many cases. Furthermore, information on the properties of the binding sites on the protein surface can sometimes be obtained. The Cotton effects constituting a CD-spectrum are obtained from chromophores bound in an asymmetric environment. Tryptophan in human serum albumin (HSA) can therefore be supposed to give characteristic internal Cotton effects in the region of about 250–300 nm and also external Cotton effects, when it is bound to HSA [7]. The purpose of the present communication is to show the usefulness of CD measurements for studies on the tryptophan binding to HSA.

2. Materials and methods

L-Tryptophan was obtained from Merck A.G., Darmstadt, and was stated to be more than 99% pure. Human serum albumin (HSA) was received as a gift from KABI, Stockholm. It was freed from fatty acids and other possibly adsorbed impurities by treatment with activated charcoal according to the method of Chen [8]. The monomer of HSA was thereafter isolated by repeated gel filtration on Sephadex G-200 and G-100 in 0.1 M KCl. The homogeneity of the monomer was checked by polyacrylamide gel electrophoresis at pH 8.3 in 7% gels.

CD-spectra were recorded with an automatic recording spectropolarimeter, JASCO J-20, Japan Spectroscopic Co., Tokyo. The instrument was calibrated with D-10-camphorsulfonic acid and tested daily with an internal test signal. The measurements were made in rectangular cells (5–20 mm pathlength) in a thermostated cellholder at 10°. The size of the cells was chosen to optimize the measuring conditions and to avoid voltages over the photomultipliers exceeding 700 V. Each sample was filtered through a Millipore filter (0.22 μ) and scanned at least 2 times. The samples were dissolved in 0.1 M KCl at pH 9.0. The ellipticity obtained is expressed as molar ellipti-

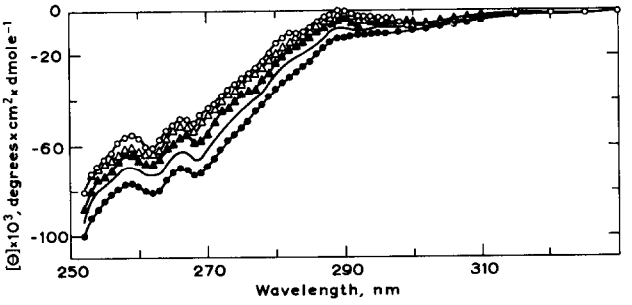

Fig. 1. Circular dichroism spectra of human serum albumin monomer (HSA) (● — ● — ●) and of HSA together with 2 molar excess (———), 6 molar excess (▲ — ▲ — ▲), 10 molar excess (△ — △ — △) and 15 molar excess (○ — ○ — ○) of tryptophan in 0.1 M KCl at pH 9.0 and 10°.

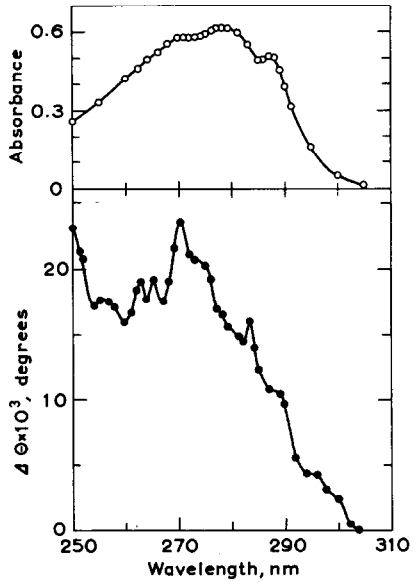

Fig. 2. Absorbtion spectrum of tryptophan at pH 9.0 in 0.1 M KCl (upper part) and CD-difference spectrum of HSA–tryptophan complex (lower part). The CD-spectrum of HSA (1.09 mg/ml) alone is subtracted from the spectrum obtained from HSA together with a 10-fold molar excess of tryptophan in 0.1 M KCl at pH 9.0 and 10°.

city, based on the amount of albumin present, the concentration of which was determined from the absorption spectrum, using $A^{1\%}_{280\ nm} = 5.80$ [9] and a molecular weight of 69,000. For the calculation of binding constants of the tryptophan–albumin complex, the primary ellipticity data (in degrees) obtained with constant amount of HSA (1.0 mg/ml) and varying amounts of tryptophan in 10 mm cells were used. The data thus obtained were corrected for the ellipticity of tryptophan itself ($(\theta) = 8.1 \times 10^2$ at 270 nm, pH 9.0 and 10° in 0.1 M KCl).

3. Results

HSA has a characteristic CD-spectrum with a negative ellipticity in the so called aromatic part of the ultraviolet wavelength region (300–250 nm). However, when L-tryptophan is added, the negative ellipticity values decrease successively as can be seen from fig. 1. The changes seen depend on the formation of a HSA–tryptophan complex and to some extent to the ellipticity of tryptophan itself. Fig. 2 presents the difference spectrum obtained after subtraction of the HSA curve from a spectrum of a sample also containing a 10-fold molar excess of tryptophan. In the same figure, the absorption spectrum of tryptophan is presented to allow a comparison to be made with the difference spectrum. As is evident, great similarities exist between the two spectra. Thus, for instance, the maximum of the difference spectrum at 270 nm coincides well with an absorption band of tryptophan giving the plateau at 270–274 nm of the tryptophan spectrum. The ellipticity values obtained at 270 nm were subsequently used for the calculation

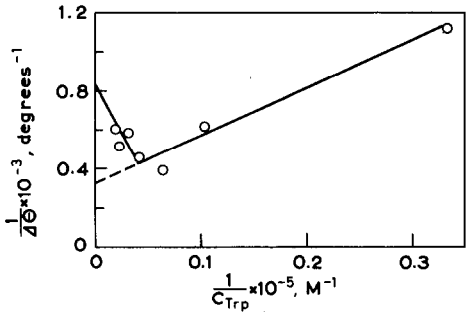

Fig. 3. A plot of the inverted ellipticities obtained of HSA–tryptophan complexes against the inverted tryptophan concentrations used. The values are related to 10 mm cells and a HSA concentration of 1.09 mg/ml in 0.1 M KCl at pH 9.0 and 10°.

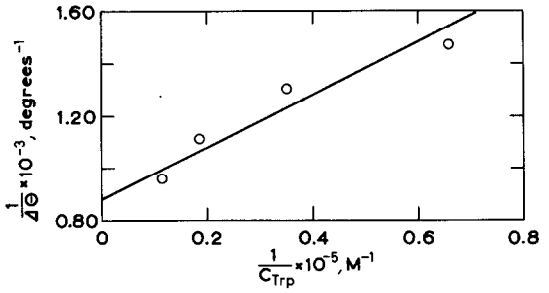

Fig. 4. A plot of the inverted ellipticities of HSA–tryptophan complexes obtained at low concentrations of tryptophan. The concentration of HSA was 1.00 mg/ml. The same conditions as in fig. 3 were used.

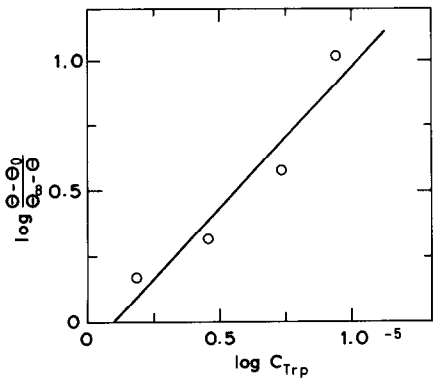

Fig. 5. A plot for calculation of the primary binding constant of the HSA–tryptophan complex. The intercept on the x-axis gives $\log k_1 = -\log C_{Trp}$. The experimental conditions used were the same as in fig. 4.

of the degree of binding of tryptophan. As free tryptophan also gives a positive ellipticity at 270 nm ($(\theta) = 8.1 \times 10^2$ degrees $\times$ cm^2 dmole^{-1} at pH 9.0 in 0.1 M KCl at 10°), the values obtained for the tryptophan–HSA complexes were corrected for the contribution from tryptophan. In fig. 3, the inverted difference ellipticity values obtained are plotted against the inverted values of the total concentration of tryptophan. Such a plot should give a straight line for each complex formed, according to Ikeda and Hamaguchi [10]. However, in the concentration interval studied (2–30-fold molar excess of tryptophan), the plot clearly reveals that tryptophan is bound to at least two different kinds of binding sites. When tryptophan is bound to the primary site a positive Cotton effect is obtained, while further binding of tryptophan yields negative Cotton effects of weaker magnitude.

The characteristics of the tryptophan binding to the primary site was studied in more detail at low concentrations of tryptophan (up to about 6-fold molar excess). It was assumed that only negligible amounts of tryptophan were bound to the secondary sites on HSA at these low concentrations. The maximal ellipticity, θ_∞, obtained when all primary sites were occupied, can be calculated from fig. 4. The intercept on the y-axis yields $1/\theta_\infty = 0.88 \times 10^3$, as determined by the method of least squares. With the albumin concentration used, θ_0 at 270 nm with no tryptophan present was -11.2×10^{-3} degrees.

In fig. 5, the logarithms of $\theta-\theta_0/\theta_\infty-\theta$ are plotted against log C_{Trp}. Such a plot gives a straight line when a ligand is bound to a homogeneous class of binding sites. The slope of the line gives the number of ligands bound and from the intercept on the x-axis the binding constant can be calculated from the equation

$$\log k = -\log C_{Lig}$$

according to Ikeda and Hamaguchi [10]. In the present case, the slope was found to be 1.077 and $k_1 = 8.06 \times 10^4$ M^{-4}, indicating that one tryptophan is bound per HSA molecule at the primary state.

When the line in fig. 3 is extrapolated to very high concentrations of tryptophan, the limit value of the ellipticity can be estimated for the situation when all binding sites on HSA are saturated. If the contribution from the primary site is subtracted, the binding constants for the binding of tryptophan to the secondary sites, *giving Cotton effects,* can be approximately estimated from a plot corresponding to the one in fig. 5. Such an estimation gave k_{sec} = about 0.4×10^4 M^{-1} and a slope between 1 and 2.

4. Discussion

The present work shows that tryptophan gives rise to Cotton effects when it is bound to HSA. These Cotton effects can be used for both quantitative and qualitative purposes. Thus, the monomer HSA contains according to our study two different binding sites. The primary site binds one tryptophan per mole protein with a binding constant of 8.1×10^4 M^{-1} at 10° and pH 9.0, while one to two molecules of tryptophan can be bound to secondary sites with an approximative binding constant of 0.4×10^4 M^{-1}. The value for the primary constant is in good agreement with the results published by McMenamy et al. [1, 4], who found values of k_1 of about $6.5–11 \times 10^4$ M^{-1} at 2–18° in 0.1 M salt. As to the binding to the secondary sites this has hitherto been characterized only incompletely due to experimental difficulties. Our values for the secondary sites should also be regarded as preliminary, but it can be concluded that the ratio between the primary and secondary binding constants is high – at least 20. King and Spencer [5] studied the binding of L-tryptophan to

to half-cystinyl bovine plasma albumin and found also that two ligands were bound to secondary sites. The ratio between the two binding constants was in their case about 40 at pH 7.95 and 24°.

The calculations made are based on the difference of the CD spectra between HSA–tryptophan complex and free HSA after correction for the ellipticity of tryptophan itself. As seen from fig. 2, the difference spectrum is similar to the absorbance spectrum of tryptophan, which strongly indicates that the Cotton effects seen for the complex originate from the tryptophan side chain. It is true that the phenyl and p-hydroxyphenyl side chains of phenylalanine and tyrosine, respectively, also absorb radiation around 270 nm, but they can most probably be excluded in this case, as the complex gives ellipticity also above 300 nm. However, it is not possible at present to conclude if the Cotton effects originate from the bound tryptophans by an asymmetric binding of the chromophore (i.e. are *extrinsic* Cotton effects) or if the tryptophan present in the HSA molecule contributes to the difference spectrum due to a changed conformation around the indol side chain (i.e. a change of the *intrinsic* Cotton effect of the tryptophan). However, as HSA contains only one tryptophan [11], at least one to two of the bound tryptophans give rise to extrinsic Cotton effects. Further binding studies after chemical modification of the tryptophan residue of HSA should give the answer if the only one tryptophan present in the HSA molecule is situated at any of the binding sites and thus can change the intrinsic Cotton effects.

Acknowledgement

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