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CHIRAL RECOGNITION BY BIOLOGICAL MACROMOLECULES

PARTIAL RESOLUTION OF RACEMIC ENONES BY ALBUMIN

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SUMMARY

A novel approach to the optical resolution of racemic enones has been introduced by using the binding properties of the transport protein albumin, which chemically binds preferentially one antipode of some α,β-unsaturated cyclic enones in a reversible manner. Simple separation of the macromolecules by ultrafiltration leads to partial resolution of racemates.

INTRODUCTION

Serum albumin is the most familiar plasma protein. It is the principal agent responsible for the transport of fatty acids and for the sequestration of physiological molecules, *viz.*, bilirubins, hormones, amino acids, etc., and also many drugs, from aspirin to antibiotics[1]. Its binding action appears to be connected to a variety of sites, a unique characteristic of this non-enzymatic, non-structural protein among the plasma proteins. Chiral interactions of bovine serum albumin (BSA) with smaller molecules were recognized some time ago; interesting examples are optical activation of the achiral bilirubin chromophore[2,3] and asymmetric induction in reactions involving prochiral substrates[4]. Recently, a commercial chiral high-performance liquid chromatographic (HPLC) column using BSA covalently bound to silica gel was introduced for the resolution of acidic and basic low-molecular-weight compounds[5–9]. We have now investigated whether novel enantiomeric enrichment approaches can be found for other substrates, based on the evident binding tendency of this transport protein.

We report here that BSA preferentially binds one antipode of some racemic α,β-unsaturated cyclic enones, and that binding is due to the formation of a reversible chemical bond. Here the BSA–enone interactions basically differ from those usually found between BSA and chromatographic analytes. As a consequence of this chemical bonding, separation of the macromolecular adducts from the low-molecular-weight species, accomplished by simple ultrafiltration, led ultimately to optically enriched samples of both (+)- and (−)-antipodes.

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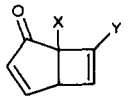
EXPERIMENTAL

Apparatus

UV measurements were performed with a Pye Unicam SP150 spectrophotometer. A Jasco J 500 C spectropolarimeter was used to measure circular dichroism (CD) spectra, from which the optical purity (OP) of the samples was obtained, OP = $|\delta\varepsilon/\Delta\varepsilon|$, $\delta\varepsilon$ and $\Delta\varepsilon$ being the CD, at some suitable maximum, of the sample and of the pure enantiomer, respectively. HPLC determinations were made with a Jasco BIP1 chromatograph equipped with a spectrophotometric detector (Uvidec 100V) and a laboratory-made data processor. A Merck RP-8 (7 μm) (25 cm × 4.6 mm I.D.) column was used with acetonitrile–water (25:75) as the mobile phase at a flow-rate of 1 ml/min and with UV detection at 225 nm. Ultrafiltrations were carried out with a Spectra/por stirred cell (S25-10) pressurized by nitrogen at about 3 atm and equipped with Spectrum C20K membranes, 20 000 daltons cutoff (Spectrum Medical Industries, Los Angeles, CA, U.S.A.).

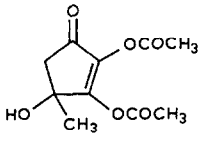
Materials

Racemic 1-methoxybicyclo[3.2.0]hepta-3,6-dien-2-one, (±)-**1**[10–12], and 7-acetylaminobicyclo[3.2.0]hepta-3,6-dien-2-one, (±)-**2**[13,14], were prepared according to the literature and 2,3-diacetyl-4-hydroxy-4-methylcyclopent-2-en-1-one, (±)-**3**, was kindly provided by Prof. M. Scotton (University of Siena). BSA, crystallized and essentially free from fatty acids, was purchased from Sigma (St. Louis, MO, U.S.A.).



1 X = OCH_3, Y = H

2 X = H, Y = $NHCOCH_3$



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RESULTS AND DISCUSSION

When the racemic bicyclic α,β-unsaturated enone (±)-**1** is dissolved in an aqueous solution of BSA, the mixture shows increasing circular dichroism with time in the range 320–430 nm. This is a spectral region where BSA is almost transparent and devoid of Cotton effects (Fig. 1a). Simultaneously, a decrease in absorbance in the same spectral region, the n → π^* absorption band of the enone and an increase in absorbance below 320 nm are observed (Fig. 1b). With molar ratios of 1:BSA ranging from 1.8 to 30, at a constant BSA concentration of $1 \cdot 10^{-4}$ *M*, time-dependent changes of the UV and CD spectra were observed in the BSA absorption spectral zone (<320 nm) up to the peptide zone (<240 nm), where variations in the ellipticity are not measurable. In particular we noted a reduction in CD in the 280–315 nm region and an increase in CD in the range 240–275 nm with respect to the CD spectrum of pure BSA solutions. In conclusion, we have a first zone, the enone (>320 nm) with no BSA chromophore, a second zone (320–240 nm), in which relatively few BSA chromo-

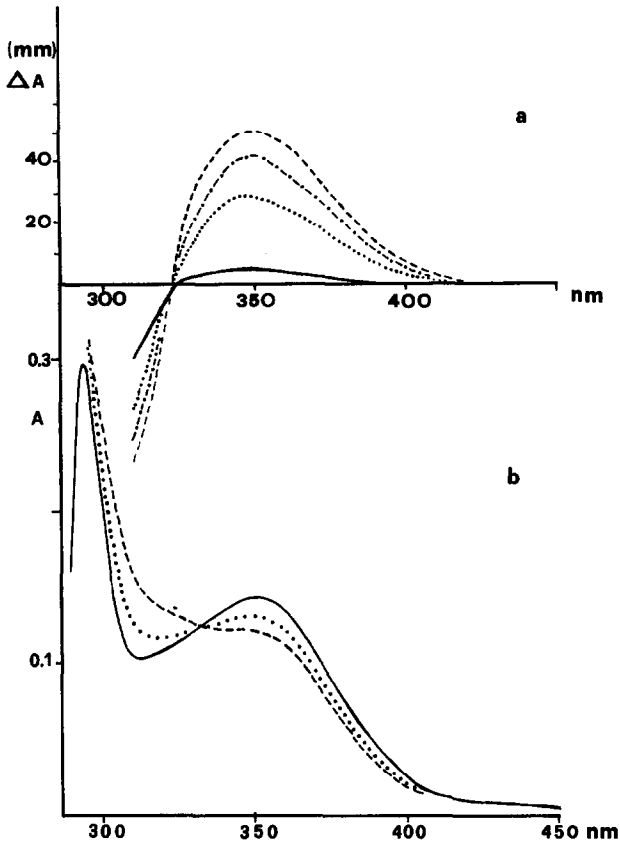

Fig. 1. CD and UV spectra after mixing **1** and BSA. (a) CD elongation 5, 60, 100 and 1100 min after dissolving 1.9 mg ($1.4 \cdot 10^{-2}$ mmol) of (±)-**1** in 9 ml of a $6.2 \cdot 10^{-5}$ *M* aqueous solution of BSA (pH 6.6). Differential circular absorbance increases with the time. (b) UV absorbance 15, 100 and 500 min after mixing. The reference cell contained a solution with the same concentration of BSA as in the sample cell. The absorbance decreases with time above 330 nm.

phores are present (48 aromatic and 17 disulphided), and a third zone (<240 nm), where the 580 peptide chromophores together with the aromatic chromophores override both in chiral and in achiral light absorption the effects due to the few enone chromophores. Note that the constancy of the CD in the spectral zone 190–240 nm implies no relevant modification of the content of α-helix and β-structure of the protein due to the binding of the enone[1]. From the variations in both the CD and UV spectra with time in the n → π* spectral region we estimate a half-life of about 1 h for the binding process at room temperature. Similar indications resulted from HPLC determinations at various times of the unbound **1** in the **1**–BSA mixtures.

Free **1** can be separated from high-molecular-weight molecules by ultrafiltration of the aqueous **1**–BSA mixture using membranes with a cut-off of 20 000 daltons. With these high-performance membranes, ultrafiltration of 10–20 ml of feed solution, with collection of 2–3 ml, is carried out rapidly (3–8 min) with respect to the reaction time.

The composition of the permeate (*i.e.*, the solution of low-molecular-weight compounds passed through the membrane) essentially reflects the composition of the feed solution, as far as free **1** is concerned, at the moment of the ultrafiltration, especially with values of the volume reduction factor (*i.e.*, the ratio of the volume of the permeate to the volume of the residual feed solution, the retentate) such as 0.2–0.4. In fact, the permeate showed a strong positive Cotton effect in the enone chromophore spectral zone (320–430 nm), owing to the enantiomeric excess of (+)-**1** present: in the same spectral region a positive CD signal was also observed in the retentate.

The enone bound to BSA can be recovered by dialysing the retentate or by dichloromethane extraction of the retentate: (−)-**1** of optical purity definitely higher than that of (+)-**1** in the permeate can thus be obtained. Hence it is clear that optical activation of **1** derives from the preferential binding of the (−)-**1** antipode to the BSA molecule. As the CD of the retentate in the enone n → π^* region is positive and in the same region there was a decrease in the absorbance after mixing **1** and BSA, we conclude that **1** bound to BSA no longer absorbs any near-UV light and therefore the observed positive CD is essentially due to the free **1** present in the retentate.

These observations indicate that binding to BSA involves a transformation of the enone chromophore. The variations in the spectroscopic properties of **1** bound to BSA and the reversible nature of the binding can be explained by the formation of a Schiff base between the carbonyl group of **1** and the ε-amino group of some lysine in the binding site of BSA. We observe that ketoimine adducts are common intermediates in the transformation of carbonyl-containing substrates catalysed by enzymes of the lysine class[15]. Alternative explanations could be the formation of a reversible Michael adduct between the enone moiety and BSA or formation of an aminoketal adduct. In any case, a shift at least of 40–50 nm of the long-wavelength absorption band towards shorter wavelengths is expected[16]. On the basis of the present data, none of these possibilities can be ruled out. Anyway, the interactions responsible for the above reaction are basically different from the weak interactions usually found under chromatographic conditions, as expected.

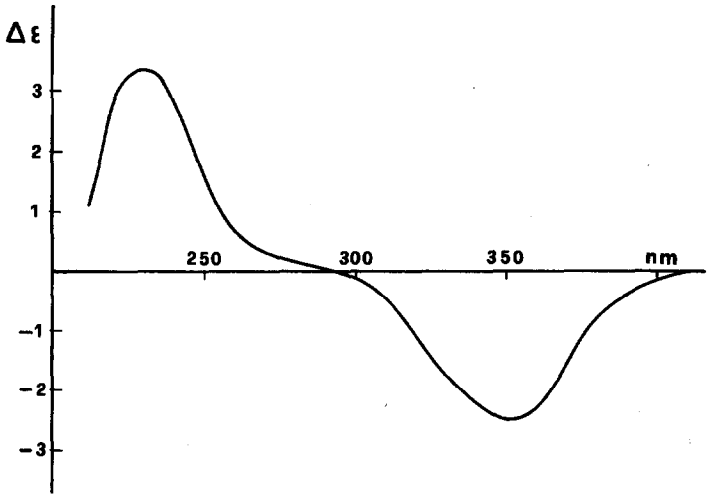

Fig. 2. CD spectrum of (−)-**1** from a sample with an optical purity of 17.1% in water ($\Delta\varepsilon$ in $l\,mol^{-1}\,cm^{-1}$).

The enantiomeric excess of (−)-**1** bound to BSA, ee^b, is obviously an important parameter. This ee, which is greater than the measured OP of (−)-**1** extracted from the overall retentate because of the free (+)-**1** present in it, can be evaluated from the measured optical purities of **1** in the permeate and in the retentate and from the mass of **1** in the permeate and the mass of free **1** in the retentate (see Appendix 1). In a typical experiment, with an initial [**1**]:[BSA] ratio of 17, [BSA] = $1.3 \cdot 10^{-4}$ and a volume reduction factor of 8.9, (−)-**1** with an optical purity of 17.1% (Fig. 2) was obtained from the retentate, whereas (+)-**1** with an optical purity of 5.1% was found in the permeate. Insertion of the experimental data in eqn. 3 (Appendix 1) gives 16% of total **1** bound to BSA. From the masses of "free" **1** in the permeate and in the retentate, the amount of bound **1** can also be obtained as the difference from total **1**; hence about 14% of **1** was bound. The above values correspond to less than three molecules of **1** bound to each molecule of BSA. From eqn. 4 (Appendix I), the enantiomeric excess of this bound **1** is about 24%. Therefore, with a [**1**]:[BSA] ratio of 17 and under the above-defined experimental conditions, the maximum OP that can be obtained for **1** is of the order of 25%. This value was effectively approached by a three-fold iteration of the process, each time dissolving fresh BSA in the permeate previously obtained.

In order to clarify as far as possible the dependence of the OP of **1** (permeate) on the ratio of [**1**] to [BSA], a set of experiments was performed under strictly controlled conditions. With a fixed BSA concentration of 7.5 mg/ml and variable amounts of **1** the data represented in Fig. 3 were obtained. With the hypothesis of n binding sites in each BSA molecule with a very high association constant we expect that at [**1**]:[BSA] ratios higher than n the optical purity of **1** in the permeate would be "diluted" because of saturation of the enantioselective sites. The experimental curve in Fig. 3, at its steepest part, gives some support to this simplified model when n is of the order of unity. In fact, experimental data, including the HPLC determinations of free **1**, are

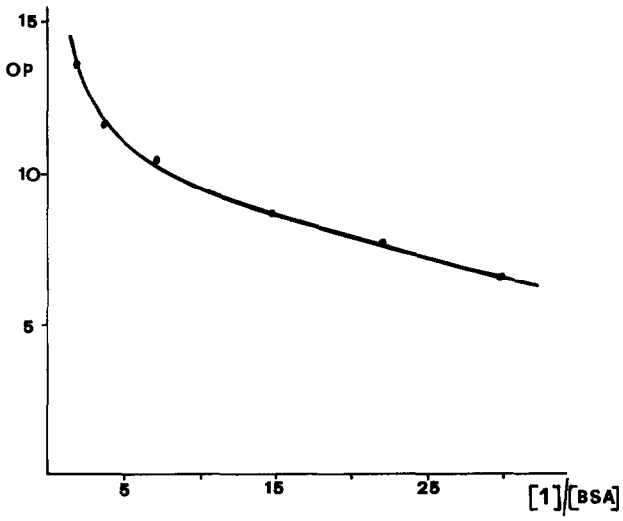

Fig. 3. Optical purity of **1** in the permeate and initial [**1**]:[BSA] molar ratio. [BSA] = $1.1 \cdot 10^{-4}$ M; ageing for 100 min at 25°C and, subsequently, 22 h at 0°C; volume reduction factor, 0.43; initial volume, 10 ml; volume of the permeate, 3 ml, obtained with 3 min of ultrafiltration.

unable to give a precise value for the number and the binding constants for each type of binding site of the protein.

More precise data, especially for small [**1**]:[BSA] ratios (0–1) would require the use of BSA purified from bound impurities and, probably the separation of monomeric from oligomeric BSA[1].

The dependence of the OP of **1** in the permeate on other variables was observed. These variables include temperature, ageing of **1**–BSA mixtures, concentration of BSA and speed of ultrafiltration. In particular, we noted a progressive degradation of **1** with ageing of **1**-BSA mixtures, especially on increasing the BSA concentration. Some reduction in the OP of **1** (permeate) was also found.

Other substrates show similar chiral interactions with BSA, as observed with the bicyclic ketone **2**, an anti-inflammatory product[13,14], and with the cyclic enone **3**. With [**2**]:[BSA] = 35 and [**2**] = $2 \cdot 10^{-3}$ *M* we observed a negative dichroism in the ketone $n \rightarrow \pi^*$ spectral band after 10 min of ageing of the proteic solution. The measured maximum of the CD at 345 nm compared with that of the pure antipode[13,14] gave an OP of 0.6%. Interestingly, in this instance the bound antipode is the positive one. For **3**, under similar experimental conditions, we observed a negative CD with a maximum at 330 nm ($\delta\varepsilon = -0.08\ \mathrm{l\ mol^{-1}\ cm^{-1}}$). In the absence of chiroptical data referring to the pure antipode, the OP of free **3** was not determined.

CONCLUSIONS

The OP of **1** ranged from 5 to 17%. Although these values are usually considered of minor interest, it should be emphasized that they are sufficient for many spectroscopic studies (*e.g.*, chiroptical determinations, chiral NMR experiments) or stereochemical correlations, the minimum OP level required being determined by the sensitivity of the instrumentation. Hence efforts to obtain higher OP are not really justified in most instances. In fact, we could measure the CD spectrum of (−)-**1** (Fig. 2) up to 200 nm, which was not possible for **1** with OP = 1%, previously obtained by partial photolysis of (±)-**1** with circularly polarized UV light[10]. However, OP for **1** greater than the above values would certainly be achieved with a substrate: BSA molar ratio of less than 1.8, as suggested by the data in Fig. 3. These data were obtained by deliberately using commercially available "pure" BSA batches. As already noted, with such low ratios the use of highly purified BSA becomes essential in order to obtain meaningful and reproducible results.

We have shown that the BSA molecule contains at least one reaction site able to discriminate the chirality of the reacting ketone **1**; probably this site would react with other ketones as, in fact, was observed with **2** and **3**. Hence the above examples partially define the possibilities of using the transport protein BSA as a chiral template for reactions involving ketones.

APPENDIX 1

Assuming no reaction other than the binding of **1** to BSA, the enantiomeric excess of **1** bound to the protein, ee^b, and that of free **1** must balance each other, and the following equation holds:

$$(m^{rf} + m^{p})OP^{p} = m^{rb}ee^{b} \qquad (A1)$$

where m^{rf}, m^{p} and m^{rb} are the masses of **1** free in the retentate, of **1** in the permeate and of **1** bound to protein, respectively, and OP^{p} is the optical purity of **1** in the permeate.

Another conservation equation is obtained by imposing optical compensation between **1** in the retentate and **1** in the permeate:

$$(m^{rf} + m^{rb})OP^{r} = m^{p}OP^{p} \tag{A2}$$

where OP^{r} is the optical purity of **1** extracted from the retentate. Then, using eqns. A1 and A2, the quantities m^{rb} and ee^{b}, not directly measured, can be expressed as functions of the experimental quantities m^{p}, m^{rf} (from HPLC), OP^{p} and OP^{r} (from CD measurements):

$$m^{rb} = \frac{m^{p}OP^{p}}{OP^{r}} - m^{rf} \tag{A3}$$

$$ee^{b} = OP^{r}OP^{p} \cdot \frac{m^{p} + m^{rf}}{m^{p}OP^{p} - m^{rf}OP^{r}} \tag{A4}$$

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