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# GENERAL APPROACH TO CHIROPTICAL CHARACTERIZATION OF BINDING OF PROCHIRAL AND CHIRAL 1,4-BENZODIAZEPIN-2-ONES TO HUMAN SERUM ALBUMIN

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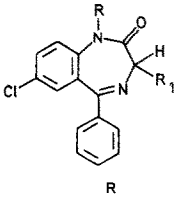
**Abstract**—General interpretation of chiroptical characteristics of binding process of prochiral (Z) and chiral (Z*) 1,4-benzodiazepin-2-ones to human serum albumin (HSA) is presented. Interpretation of binding of Z studied by circular dichroism (CD) measurements allows us to conclude that the CD within the bands of bound Z is mainly due to the chirality of the first sphere and that the greatest part of **3** is bound in the M-conformation. For Z* we conclude that (*S*)-**1** binds on both binding sites of HSA in the M-conformation, while (*R*)-**1** binds on two different binding sites in opposite conformations. Furthermore we conclude that binding via two nitrogens of the benzodiazepine, i.e. at two ends of the chromophoric system is rather more probable than via ring A.

CD-Investigations of 1,4-benzodiazepines bound to HSA have been done by two groups of authors [1–8]. The most important conclusions given by Müller and Wollert [3–5, 7, 8] can be summarized as follows: (a) all achiral [in most cases prochiral at C(3) position] 1,4-benzodiazepines bind only to one and the same binding site at the molecule of human serum albumin, (b) their binding affinities to this binding site are very different, and the annelated benzene ring A seems to be one of the essential groups for binding. Hitherto only one example of stereospecific binding of the enantiomers of a chiral 1,4-benzodiazepine derivative, (+)- and (−)-**1**, has been studied [8]. This work has revealed some differences in binding behaviour of enantiomers to HSA compared to binding to bovine serum albumine (BSA). Regarding stereospecificity of binding to HSA the following conclusions have been drawn by these authors [8]: (a) both enantiomers bind to one binding site, but their binding constants differ very much, (b) both enantiomers bind in the same absolute conformation, (c) no ligand-induced alteration of the protein conformation occurs. Sjödin *et al.* [6] came to similar conclusions about the binding of 1,4-benzodiazepines to HSA. Earlier results obtained using CD in monitoring the binding of 1,4-benzodiazepines have been discussed in terms of contribution of their 'intrinsic' and 'extrinsic' Cotton effects, using the same terms for the macromolecule, as well [7, 8]. In addition, terms like 'intrinsic', 'extrinsic' or 'induced extrinsic' and 'biphasic extrinsic' Cotton effects have been used in discussing the binding of both prochiral and chiral 1,4-benzodiazepines [3, 6–8], sometimes in rather different contexts. To avoid presumable misunderstanding in this area we in our approach to the interpretation of chiroptical data obtained for binding of prochiral and chiral 1,4-benzodiazepines use generally accepted terms [9]. Furthermore, as CD is determined only by 'absolute' arrangement of atoms and bonds around the chromophore, we use here the term 'absolute conformation' for describing molecular geometry [10]. This term can then also be applied to configurational achiral molecules, which can, however, exist only in stable chiral conformations which are separated by an energy barrier.




| | | R | $R_1$ |
|---|---|---|---|
| (S)- and (R) | 1 | H | $CH_3$ |
| | 2 | $CH_3$ | H |
| | 3 | H | H |
| (+)- and (-) | 4 | H | $OCOCH_2CH_2COOH$ |

Formulae **1**–**4**.

## DISCUSSION

In the previous paper [11] experimental results obtained for enantiomeric pairs (*S*)-/(*R*)-**1**, as well as for the prochiral compounds **2** and **3**, were presented and the interpretation of the chiroptical features of these benzodiazepine–HSA complexes is based on a generally accepted theory of protein–ligand [12] (or enzyme–substrate [13]) interaction. In the following

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discussion prochiral* benzodiazepines will be abbreviated by Z, and chiral ones by Z*.

(A) *Complexes of HSA with prochiral benzodiazepines*

If $C^0_{HSA}$ is the total molar concentration of HSA present, and $C^0_Z$ the total concentration of 1,4-benzodiazepine, and if $q$ ($0 \leqslant q \leqslant 1$) stands for the fraction of HSA which is complexed in the presence of Z then the concentrations of free HSA, complex, and free 1,4-benzodiazepine, respectively, are:

$$(1 - q)C^0_{HSA},\ qC^0_{HSA},\ \text{and}\ C^0_Z - qC^0_{HSA}.$$

Using differential absorbance $\Delta A \equiv A_L - A_R$ and the definition of $\Delta\varepsilon$ according to equation (1)

$$\frac{\Delta A}{d} = C\Delta\varepsilon \tag{1}$$

the measured value $(\Delta A/d)_{meas.}$ will then be ($d$: cell thickness in cm):

$$\left(\frac{\Delta A}{d}\right)_{meas.} = (1 - q)\cdot C^0_{HSA}\Delta\varepsilon_{HSA} + qC^0_{HSA}\Delta\varepsilon'_{HSA} + qC^0_{HSA}\Delta\varepsilon_{Z,\,compl.} + qC^0_{HSA}\Delta\varepsilon^{\mu,\mu}_{Z\leftarrow HSA}. \tag{2}$$

The first term comes from free HSA and the second from the chromophores of HSA in the complex. We have, of course, to substitute an unknown value $\Delta\varepsilon'_{HSA}$ for $\Delta\varepsilon_{HSA}$, the latter of which is valid for the free serum albumin, because there may be alterations of this CD caused by the $\mu,\mu$ mechanism [14] ('dipole–dipole interaction', induction of optical activity within the absorption bands of HSA due to perturbation from the $\pi$-system of benzodiazepine) and because of possible changes of conformation of the protein by ligand binding. The third term describes the inherent optical activity of the complexed Z molecule, because of the preference of one chiral conformation of Z when bound to HSA. Finally the last term describes the induced CD within the absorption bands of Z, caused again by chiral perturbation of its chromophores by $\pi$-system of HSA (e.g. according to the $\mu,\mu$ mechanism). In this description we neglect any chiral perturbation of free Z molecules in solution by optically active 'solvent' (i.e. by the chiral surrounding with HSA molecules). We can modify equation (2) by making the following assumption, which seems reasonable from the point of view of enzyme and protein chemistry; vic. prochiral benzodiazepines are bound specifically to HSA, i.e. to the same sites and in the same absolute conformations as their chiral analogues. It is, therefore, possible to use the chiroptical data of a chiral benzodiazepine (Z*) instead of these of HSA-bound achiral ones (Z).

From molecular models and from the X-ray structure [15, 16] we know that the seven membered ring (B) of diazepam (typical prochiral 1,4-benzodiazepine) deviates very much from a planar arrangement, and the same holds for similar prochiral and chiral molecules [17, 18]. This must also be true for the bound form, thus because of this noncoplanarity of the full chromophoric system the latter is inherently chiral [9]. Based on general experience we can safely assume that the CD within the bands of bound prochiral benzodiazepines is mainly due to inherent chirality, i.e. chirality of the first sphere [9]. This is in general [9] one or two orders of magnitude larger than the CD induced within the same molecule by the chiral environment of the protein, i.e.

$$\Delta\varepsilon_{Z,\,compl.} \gg \Delta\varepsilon^{\mu,\mu}_{Z\leftarrow HSA}.$$

We will thus neglect the last term in equation (2).

From the value measured for the solution containing the complex we subtract the value measured for free HSA (same molar concentration† and the same cell thickness). This difference we indicate by small $\delta$ and (2) then reads:

$$\delta\left(\frac{\Delta A}{d}\right)_{meas.} \equiv \left(\frac{\Delta A}{d}\right)_{meas.} - C^0_{HSA}\Delta\varepsilon_{HSA} \approx qC^0_{HSA}(\Delta\varepsilon'_{HSA} - \Delta\varepsilon_{HSA}) + qC^0_{HSA}\Delta\varepsilon_{Z,\,compl.}. \tag{3}$$

$\Delta\varepsilon'_{HSA}$ is also composed of (at least) two terms, one describing the CD in that conformation which is present in the complex (and which must not be identical with that of HSA alone), and one coming from the interaction of the chromophores of HSA (amide groups, aromatic rings, —*S*—*S*— bridges) with the $\pi$-system of the bound ligand. As these two terms depend, however, on each other we cannot split $\Delta\varepsilon'_{HSA}$ similarly into two summands.

The CD of a free chiral benzodiazepine (Z*) can be described in similar manner [11]. It will be the sum of contributions from different spheres and again we can neglect contributions from others, if the first one is chiral [9]. NMR studies have shown‡ that the seven membered ring of chiral 1,4-benzodiazepines is quite rigid in a number of solvents at 35 °C, and present practically only in one conformation, while prochiral 1,4-benzodiazepines possess different degrees of conformational mobility depending on the N(1) substitution and on the solvent [19–21].

A chiral first sphere present in Z* should lead to relatively strong CD within the different bands, as is indeed found [11]. We can therefore describe the CD of a free chiral 1,4-benzodiazepine quantitatively by the CD of its chiral first sphere alone, and neglect contributions from other spheres. This gives us the possibility of checking the above assumption about specific binding in the complex. As HSA does not show absorptions and CD above 300 nm we concentrate for the moment on the Cotton effect around 313 nm. $\delta(\Delta A/d)_{meas.}$ for the complex between HSA and **2** or **3** is quite large over the whole wavelength

* 'Prochiral' and 'chiral' in this context refer always to the configuration, because actually also for 'prochiral' Z the sevenmembered ring can adopt only one of two enantiomeric stable chiral conformations.

† For proteins it is customary to calculate the CD per amino acid unit using the average molecular weight of approx 110 for one residue. Here, as we are dealing with the CD-properties of complexes between 1 molecule of protein and 1 or more molecules of ligand, we prefer to use molar concentrations based on the molecular weight 69,000 for HSA.

‡ Unpublished results from Department of Biomedical and Biochemical Research, CRC.

range; e.g. at a concentration of $1.44 \times 10^{-5}$ M we [11] found $-0.76 \times 10^{-4}$ for **2** and $-0.61 \times 10^{-4}$ for **3**, respectively at 313 nm; the corresponding value for the chiral benzodiazepine (*S*)-**1** is $-1.36 \times 10^{-4}$. Such large values for the complexes of **2** and **3** (approx 50 per cent of that of the chiral compound) would not be consistent with unspecific binding of prochiral benzodiazepines, i.e. an equal binding in both possible conformations.

In order to discuss these data for bound achiral benzodiazepines quantitatively we assume for the moment that fractions $q_M$ and $q_P$ of the bound Z are complexed in the same absolute conformations as are adopted by the chiral molecules (*S*)-**1** (M) and (*R*)-**1** (P), respectively (see Fig. 1). This leads to

$$\delta\left(\frac{\Delta A}{d}\right)_{meas.} \approx qC^0_{HSA}(\Delta\varepsilon'_{HSA} - \Delta\varepsilon_{HSA}) + qC^0_{HSA}(q_M\Delta\varepsilon^S_{Z^*} + q_P\Delta\varepsilon^R_{Z^*}) \quad (4)$$

where $\Delta\varepsilon^S_{Z^*}$ and $\Delta\varepsilon^R_{Z^*}$ are the CD-values for chiral (*S*)-**1** and (*R*)-**1**, respectively, $q_M + q_P = 1$, and using the above mentioned assumption that the CD of complexed Z can be approximated by the CD of free Z*, since also for such bound prochiral 1,4-benzodiazepines the CD is mainly determined by the chirality of their first spheres. Making use of the equality

$$\Delta\varepsilon^R_{Z^*} = -\Delta\varepsilon^S_{Z^*} \quad (5)$$

(4) then becomes

$$\delta\left(\frac{\Delta A}{d}\right)_{meas} \approx qC^0_{HSA}(\Delta\varepsilon'_{HSA} - \Delta\varepsilon_{HSA}) + q(2q_M - 1)\,C^0_{HSA}\cdot\Delta\varepsilon^S_{Z^*}. \quad (6)$$

The CD of a solution containing free and complexed (with Z) HSA can thus be described by $\Delta\varepsilon^S_{Z^*}$ of the *S*-configurational enantiomer, and $q_M$, the fraction of Z bound in the M-conformation.

Concentrating again on the Cotton effect above 300 nm equation (6) further simplifies to

$$\delta\left(\frac{\Delta A}{d}\right)^{313}_{meas.} \approx q(2q_M - 1)C^0_{HSA}\Delta\varepsilon^S_{Z^*} \quad (7)$$

because $\Delta\varepsilon_{HSA}$, and therefore also $\Delta\varepsilon'_{HSA}$ is already zero for these wavelengths.

Using the values for $q$ from the gel filtration studies [11], where also was found $\Delta\varepsilon^S_{Z^*} = -9.47$ the fractions $q_M$ are calculated to amount from 0.85 to 0.95. Within the accuracy of our CD-measurements these figures mean that either all or at least the greatest part of **3** is bound in the M-conformation.

(B) *Complexes with chiral benzodiazepines*

Using the same assumption and notations as before we obtain for the differential absorbance of a solution containing HSA and chiral 1,4-benzodiazepine Z*:

$$\left(\frac{\Delta A}{d}\right)_{meas.} = (1-q)C^0_{HSA}\Delta\varepsilon_{HSA} + qC^0_{HSA}\Delta\varepsilon'_{HSA} + qC^0_{HSA}\Delta\varepsilon_{Z^*,compl.} + qC^0_{HSA}\Delta\varepsilon^{\mu,\mu}_{Z^*\leftarrow HSA} + (C^0_{Z^*} - qC^0_{HSA})\Delta\varepsilon_{Z^*}. \quad (8)$$

In this equation we had to add one more term to those of equation (2) which describes the optical activity of uncomplexed 1,4-benzodiazepine Z* in solution.

Although for a chiral Z* the barrier for ring inversion should be even higher than for prochiral Z, and in solution only one conformer is present [22], we cannot exclude that the energetically unfavoured conformation of the seven membered ring is stabilized in the complex by binding to HSA (e.g. by partial rehybridization within the amide or/and the C=N moiety). It is known [11] that actually both enantiomers, (*S*)-**1** and (*R*)-**1**, are bound to HSA, but the (*R*)-form only to a much lesser extent than the (*S*)-form. Furthermore, we know from gel filtration experiments [11] that there exist two binding sites for (*S*)-**1** on the protein, and one or two for (*R*)-**1**. As we have shown above, the main binding site for prochiral **3** accepts the molecules in the M-conformation, it is therefore reasonable to assume that the main portion of (*S*)-**1** is bound at this same site and also in its energetically preferred M-conformation. Binding on the second site could, however, be either in the same M- or in the P-conformation. We obtain thus for the Cotton effects of a solution of the complex between (*S*)-**1** and HSA

$$\delta\left(\frac{\Delta A}{d}\right)^S_{meas.} = qC^0_{HSA}(\Delta\varepsilon'_{HSA} - \Delta\varepsilon_{HSA}) + q[q_M\Delta\varepsilon^S_{Z^*} + q_P\Delta\varepsilon^R_{Z^*}]C^0_{HSA} + (C^0_{Z^*} - qC^0_{HSA})\Delta\varepsilon^S_{Z^*} + qC^0_{HSA}[q_M\Delta\varepsilon^{S,\mu,\mu}_{Z^*\leftarrow HSA} + q_P\Delta\varepsilon^{R,\mu,\mu}_{Z^*\leftarrow HSA}]. \quad (9)$$

As the first sphere is chiral and determines practically completely the CD, we can neglect the contribution of the methyl group at the centre of chirality to the CD and have used, therefore, the same values $\Delta\varepsilon^S_{Z^*}$ and $\Delta\varepsilon^R_{Z^*}$ throughout this discussion for the chiral and prochiral bound molecules. Concentrating for the moment on the region above 300 nm the first term of (9) drops out, and further simplification as for the prochiral case leads to

$$\delta\left(\frac{\Delta A}{d}\right)^{S,313}_{meas.} = [2q(q_M - 1)C^0_{HSA} - C^0_{Z^*}] \times \Delta\varepsilon^S_{Z^*} + qC^0_{HSA}(q_M\Delta\varepsilon^{S,\mu,\mu}_{Z^*\leftarrow HSA} + q_P\Delta\varepsilon^{R,\mu,\mu}_{Z^*\leftarrow HSA}). \quad (10)$$

Figure 2. of the previous paper [11] shows that, e.g. at a $5.00 \times 10^{-5}$ M concentration, the CD above 300 nm is practically identical for free (*S*)-**1** and for its complex with HSA. Inserting these values into equation (10) leads then to

$$q_M = \frac{(2 - r^R)}{(2 - r^R) - r^S} \quad (11)$$

if the ratios $\Delta\varepsilon^{S,\mu,\mu}_{Z^*\leftarrow HSA}/\Delta\varepsilon^S_{Z^*}$ and $\Delta\varepsilon^{R,\mu,\mu}_{Z^*\leftarrow HSA}/\Delta\varepsilon^S_{Z^*}$ are called $r^S$ and $r^R$, respectively. As the induced CD will not amount to more than at maximum a few per cent, $q_M$ is thus practically equal to 1. For a $1.45 \times 10^{-5}$ M concentration the curves for (*S*)-**1** in the free and in the complexed form deviate by approx 15 per cent from each other (cf. Fig. 1. of previous paper) the experimental error of the CD-measurements [11] make this deviation, however, not significant. From

the CD-results one can thus conclude that most probably on both binding sites of HSA for (*S*)-**1** these molecules are bound in the M-conformation.

Similar equations can be written for the complex with (*R*)-**1** which read then

$$\delta\left(\frac{\Delta A}{d}\right)^{R}_{\text{meas.}} = qC^{0}_{\text{HSA}}(\Delta\varepsilon'_{\text{HSA}} - \Delta\varepsilon_{\text{HSA}}) + [2q(q_{\text{P}} - 1)C^{0}_{\text{HSA}} + C^{0}_{Z^*}]\Delta\varepsilon^{R}_{Z^*} + qC^{0}_{\text{HSA}}(q_{\text{M}}\Delta\varepsilon^{S,\mu,\mu}_{Z^*\leftarrow\text{HSA}} + q_{\text{P}}\Delta\varepsilon^{R,\mu,\mu}_{Z^*\leftarrow\text{HSA}}) \quad (12)$$

and

$$\delta\left(\frac{\Delta A}{d}\right)^{R,313}_{\text{meas.}} = [2q(q_{\text{P}} - 1)C^{0}_{\text{HSA}} + C^{0}_{Z^*}]\Delta\varepsilon^{R}_{Z^*} + qC^{0}_{\text{HSA}}(q_{\text{M}}\Delta\varepsilon^{S,\mu,\mu}_{Z^*\leftarrow\text{HSA}} + q_{\text{P}}\Delta\varepsilon^{R,\mu,\mu}_{Z^*\leftarrow\text{HSA}}). \quad (13)$$

If (*R*)-**6** would be exclusively bound in the energetically favoured P-conformation equation (13) becomes

$$\delta\left(\frac{\Delta A}{d}\right)^{R,313}_{\text{meas.}} = C^{0}_{Z^*}\Delta\varepsilon^{R}_{Z^*} - qC^{0}_{\text{HSA}}\Delta\varepsilon^{R,\mu,\mu}_{Z^*\leftarrow\text{HSA}}. \quad (14)$$

For $C^{0}_{Z^*} = C^{0}_{\text{HSA}} = 1.45 \times 10^{-5}$ M we found [11] $q = 0.18$ and the CD deviates from that of the free ligand by approx 30 per cent, which is definitely outside the margin of error of our measurements. With this value one obtains for the ratio $\Delta\varepsilon^{R,\mu,\mu}_{Z^*\leftarrow\text{HSA}}/\Delta\varepsilon^{R}_{Z^*}$ a value of $-1.8$ which would mean that the induced CD (chirality of the third sphere) should be at least as large as the inherent CD (chirality of the first sphere). Such a magnitude for an induced CD is far beyond each experience in CD-spectroscopy, and this result is then inconsistent with the assumption of $q_{\text{P}} = 1$, i.e. exclusive binding of (*R*)-**1** in the P-conformation. CD is thus in favour of two different binding sites also for (*R*)-**1**.

The binding free energy of HSA for (*S*)-**1** ranges from 27 to 35 kJ/mol (6.4 to 8.3 kcal/mol), depending on the binding site [11], for (*R*)-**6** the respective value is in the range from 17 to 31 kJ/mol (4.0–7.5 kcal/mol). In Fig. 1. two diastereomeric conformations of (*S*)-**1** are represented; the M-conformation possesses the lower energy. Other factors remaining constant, this

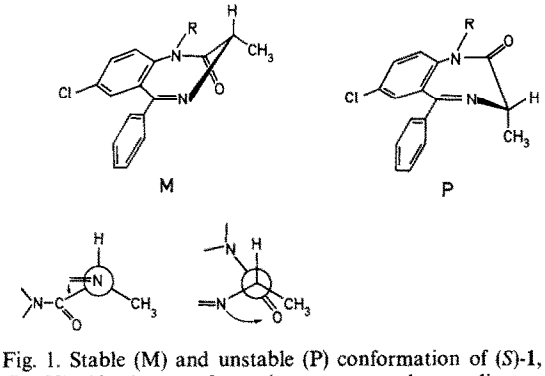


Fig. 1. Stable (M) and unstable (P) conformation of (*S*)-**1**, (R=H). Absolute conformations are named according to Cahn, Ingold and Prelog [24]. For the more stable conformation (left) both torsion angles along the 2,3- and 3,4-bond are negative, so it can be assigned the M-conformation. For the less stable conformation (right) the descriptor is then P.

latter difference should reflect the free energy difference between the P- and M-conformation of (*R*)-**1**. Our data are in agreement with the fact that the M-conformer of (*R*)-**1** is of higher steric energy than the P-conformer. The high inversion barrier found by NMR-technique [19–21] seems to contradict such an inversion of ring chirality, if, however, during binding to HSA the benzodiazepine is protonated (at the azomethine or/and the amide moiety) by a nearby proton source in the protein then this barrier will be appreciably lowered.

Our CD-data allow also some conclusions about the binding site on the benzodiazepine. As the CD curves within the 313 nm band, which can be ascribed to the $B_{2u}$-transition of the partial chromophore formed by ring A and the C=N group [23] is only weakly changed by complexing, it is rather improbable that HSA binds a benzodiazepine mainly via the $\pi$-system of ring A, as has been assumed by Müller and Wollert [2, 3]. In their discussion of the CD they consider only induced Cotton effects in the benzodiazepine molecule by the chiral surrounding of complexing HSA, but do not take into account the much stronger effects of the chiral first sphere, which is conformationally fixed in the complex even for the prochiral derivatives. Strong interactions with the $\pi$-system of ring A would perturb this latter appreciably and should then more drastically change the CD-spectrum of benzodiazepines in the bound form. Binding via the two nitrogens of the benzodiazepine, i.e. at the two ends of the chromophoric system, is rather more probable on the basis of the CD results.

In our discussion of the origin of the different CD-bands of chiral benzodiazepines we concluded [14, 23] that the Cotton effect around 286 nm corresponds to the $B_{2u}$-transition of the second partial chromophore formed by the phenyl ring and the C=N moiety. During binding the benzodiazepine molecule may well be forced to adopt another torsion angle around the bond connecting the phenyl substituent with the seven membered ring. In the CD-spectra of complexed (*S*)-**1** and (*R*)-**1** the greatest deviation from the shape of the CD-curve of the free ligands is indeed found around 285 nm which strongly supports our band assignment [23].

This treatment of the CD of complexes between HSA and 1,4-benzodiazepines as presented here should actually be of general validity and could be applied to describe the chiroptical properties of any other similar system of protein–ligand interaction with prochiral and chiral substrates. The benzodiazepine case, is, however, especially favourable because of the strong CD-bands due to inherent chirality of the first sphere.

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