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A Second-Order Asymmetric Transformation of Racemic 2-Hydroxymethyl[5]thiaheterohelicene into a Single Enantiomer upon Uptake by Bovine Serum Albumin

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Abstract: Racemic 2-hydroxymethylthieno[3,2-e:4,5-e']di[1]benzothiophene (2-HT) with a labile helical structure was converted into a *P* enantiomer upon uptake by bovine serum albumin (BSA) in 1 % ethanol–water. The effect of the alteration of the molar ratio [2-HT] : [BSA] on UV and CD spectra of the 2-HT–BSA complex solution revealed that BSA possesses two different substrate-binding sites with a distinct ability to recognize chirality of enantiomers of 2-HT. The stability of the 2-HT–BSA complex and the chirality recognition at the two sites of BSA were evaluated by the equilibrium constants and the thermodynamic parameters which were obtained from the temperature-dependence of CD-absorptional intensities. BSA pretreated at 50 ~ 70 °C in an aqueous solution exhibited a great depression of ability in discriminating chirality between enantiomers of 2-HT, though it possessed slightly altered ability for uptake of 2-HT, in comparison with untreated BSA. Copyright © 1996 Elsevier Science Ltd

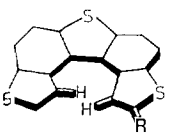
INTRODUCTION

A second-order asymmetric transformation (SAT) in which a racemate interacts with a chiral agent so as to be converted into a single enantiomer, seems to be an appealing phenomenon from the viewpoint of a practical application to an asymmetric synthesis[1] and an analogy with biological systems.[2] The SAT may occasionally happen, provided that enantiomers of a racemate are racemizable at an appropriate rate and, in addition, the produced diastereomeric complex with a chiral agent can be removed from the reaction system.[3] So far, the examples of this phenomenon have been substantially confined to the difference in crystallization behaviors of diastereoisomeric complexes: most instances being found among salt formations between chiral acids and chiral bases.[4] A sole instance has been found as a formation of a charge transfer complex between racemic heterohelicene and a chiral acceptor, 2-(2,4,5,7-tetranitro-9-fluorenylidene-amino-oxy)propionic acid (TAPA).[5] As we recently noted that the SAT phenomenon had been encountered upon uptake of thiaheterohelicene by bovine serum albumin (BSA),[6] we describe herein more detailed studies on the interaction between the components involved.

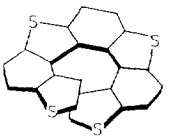
The serum albumins have been recognized as the principal proteins in plasma, and they contribute significantly to the transport, distribution and metabolism of a wide variety of endogenous and exogenous substrates.[7] In particular, BSA has found wide use as a resolving agent for racemic substrates in both homogeneous[8] and heterogeneous[9] phases, owing to its ability to discriminate between the enantiomers of a racemic mixture. However, the behavior of BSA which is mentioned here differs explicitly from that in a resolution in which the ratio between enantiomers of a racemic substrate remains unchanged. Recently, it has

been reported that the chirality of racemic 1,1'-bi-2-naphthol complexed to BSA was partially biased (not totally changed) upon near-UV irradiation, providing the enrichment of the *R*-enantiomer of the substrate.[10]

The molecular shape of 2-hydroxymethylthieno[3,2-e:4,5-e']di[1]benzothiophene (2-hydroxymethyl-[5]thiaheterohelicene, 2-HT) investigated in this study, which belongs to a helicene family, is helical on account of the steric repulsion between the terminal hydrogens. The crystal structure of the compound has not been reported yet, but may be anticipated to consist of stable *P* and *M* enantiomers by analogy with the result of the X-ray analysis of [5]TH.[11] The intramolecular inversion of the helix, however, occurs easily in solution, causing so rapid a racemization that the enantiomers cannot be resolved at room temperature.[12] Thus, a solution of 2-HT gives no absorption peaks in its CD spectrum. In the thiaheterohelicene series, an increase in the number of the rings makes the racemization rate slower and, at last, bisthieno[3',2':4,5]benzo[1,2-b:4,3-b']di[1]benzothiophene ([7]TH) possessing a skeletal overlap between the intramolecular terminals[13] manifests no apparent racemization at room temperature.[12] Consequently, racemic [7]TH can be readily resolved into P and M enantiomers by using an HPLC column of silica-gel linked with optical active TAPA.[14]



[5]TH R = H
2-HT R = CH_2OH



[7]TH

RESULTS AND DISCUSSION

Uptake of 2-HT by BSA. The compound 2-HT is practically insoluble in water and only slightly soluble (less than 1.7×10^{-7} mol dm^{-3} as estimated by the UV-absorptional intensity of its saturated solution) in 1 % ethanol–water of the present solvent system. An equimolar mixture of 2-HT and BSA with concentration of 1.53×10^{-5} mol dm^{-3} in 1 % ethanol–water solution was incubated by gentle stirring for *ca.* 30 min at 25 ± 1 °C to give a transparent solution. Figure 1 shows the UV absorption spectrum of the solution of r = 1, where r is the concentration ratio [2-HT] : [BSA], together with the reference spectra of BSA and 2-HT. The complexation does not fundamentally alter the shapes and the maximum wavelengths of both 2-HT and BSA spectra, except for a slight broadening and a little bathochromic effect by 1~4 nm for each absorption band. Further, it was ascertained that addition of 1 % ethanol to an aqueous solution of BSA brings about no alteration of either the UV or CD spectra within the range of wavelengths measured in this study.[15]

In contrast to the slight alterations of the UV spectra, drastic changes appeared in the CD spectra of the 2-HT-BSA complex solution (Fig.2), particularly in the longer wavelength region (280~400 nm) where BSA has no absorption maxima. Individual CD absorption bands turning up in this region are well resolved, showing good correspondence of the maximum wavelengths to those of the UV absorption bands of 2-HT-BSA and 2-HT. Comparison of these intense absorptions with the CD absorptions of (*P*)-[7]TH in $CHCl_3$ (Fig.2, (c)) demonstrates the existence of three major identified absorptions; α, p, and β bands according to the Clar's nomenclature.[16] It may be found that these three bands exhibit the same signs of the Cotton effects as those of the corresponding absorptions of (*P*)-[7]TH,[17] respectively: the α band has a negative sign, and the p and β bands have positive ones. These facts indicate clearly that the appearance of the new peaks in the CD spectrum of the 2-HT-BSA complex solution is derived from the chirality of a 2-HT molecule, the absolute configuration of which may be fixed definitely as a *P* form upon uptake by BSA.

In order to investigate the maximum uptake of 2-HT per one molecule of BSA, a mixture containing 2-HT and BSA (5:1) in 1 % ethanol–water was incubated for 30 hours. Then, after removing the insoluble excess 2-

HT by filtration, the UV absorption of the filtrate was measured to show that the uptake of 2-HT does not exceed 2 mole equivalents for BSA. Furthermore, when the aqueous solution of the 2-HT-BSA complex was extracted with an equal volume of diethyl ether at room temperature, 2-HT was quantitatively recovered in the ether phase which gave no CD absorptions. Addition of 2-HT to the extracted aqueous phase containing BSA yielded the same CD absorption peaks at the same wavelengths as those of the original 2-HT-BSA solution. These facts imply that the uptake of 2-HT by BSA is completely reversible and the interaction between both components seems to be too weak to withstand extraction by ether, but is strong enough to prevent a 2-HT molecule from undergoing inversion of its helix.

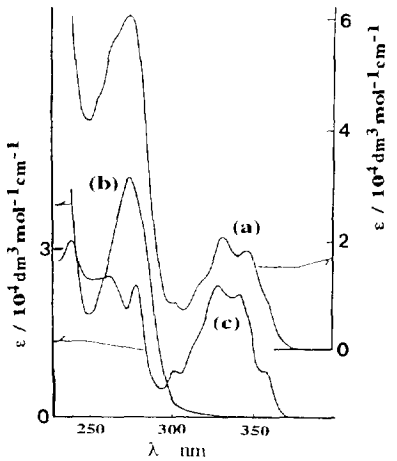


Fig. 1 UV spectra of an aqueous 2-HT-BSA solution of r = 1 (a, [2-HT-BSA] = 1.53 x 10^{-5} mol dm^{-3}), an aqueous BSA solution (b, [BSA] = 1.53 x 10^{-5} mol dm^{-3}), and 2-HT in EtOH (c, [2-HT] = 1.53 x 10^{-5} mol dm^{-3}).

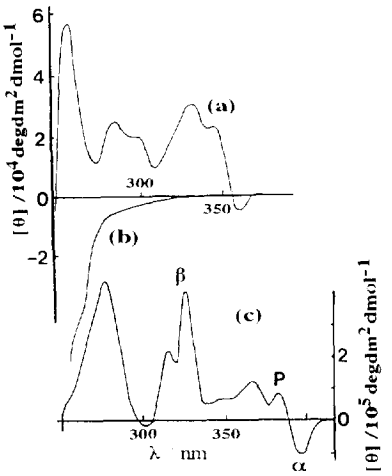


Fig. 2 CD spectra of an aqueous 2-HT-BSA solution of r = 1 (a, [2-HT-BSA] = 1.53 x 10^{-5} mol dm^{-3}), an aqueous BSA solution (b, [BSA] = 1.53 x 10^{-5} mol dm^{-3}), and (*P*)-[7]TH in $CHCl_3$ (c, [(*P*)-[7]TH] = 8.34 x 10^{-5} mol dm^{-3}).

Uptake Sites of BSA and Their Chirality Recognition. As the maximum amount of the 2-HT uptake was found to be 2 mole equivalents for BSA, the variation of the intensities of the UV and CD absorptions of the 2-HT-BSA complex was examined with respect to the change in the molar ratio r from 0.2 to 2.0 while keeping a BSA concentration constant. The intensity of the UV absorptions of the 2-HT-BSA solution increased linearly with an increase of the r value up to 2 (Fig.3). On the other hand, as for the changes in the CD-absorptional intensities, the slope of the linear plots differed between the ranges of 0~1 and 1~2 of r, the slope of the former being steeper than that of the latter (Fig.4). The increment of the apparent ellipticity (the ordinate of Figure) from the r value of 1 to 2 was 3.24 m deg that amounts to *ca.*73 % in comparison with the increment of 4.42 m deg from the r of 0 to 1. This difference between the changes of the UV and CD absorptions against the variations in r may be explicable in terms of the number of the substrate-binding sites in BSA and their chirality recognition. Namely, BSA possesses two uptake sites (1 and 2) with different abilities to discriminate between the chiralities of the enantiomers of 2-HT, which agrees with the existence of the two substrate-binding domains in human serum albumin (HSA) as determined by X-ray crystallography.[18] Therefore, it may be assumed that one uptake site of BSA bears lower chiral discrimination ability by about 27 % than the other uptake site.

When an adequate quantity of BSA was dissolved in an aqueous solution of the 2-HT-BSA complex of r = 2 leading to a solution of r = 1, followed by the incubation for one day, the apparent molecular ellipticity of each

absorption band was then enhanced *ca.* 16 %. If the incubation time was no longer than 30 min, the enhancement of the ellipticity remained *ca.* 10 %. It was ascertained that further addition of BSA to the solution containing only BSA resulted in almost no change in the longer wavelength region measured for the 2-HT-BSA complex. In a 2-HT-BSA solution of r = 2, both site 1 (provisionally with a higher ability for chiral discrimination) and 2 (with a relatively lower ability) are anticipated to be coordinated by 2-HT. Further addition of BSA to this solution then compels 2-HT at site 2 to migrate gradually to unoccupied site 1, raising the CD intensities in accord with the difference of abilities in discriminating chirality. It is thought that the migration of 2-HT from site 2 to unoccupied site 1 may be considerably slow, compared with the initial uptake of 2-HT by BSA.

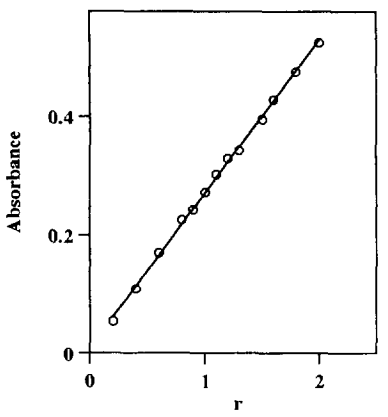


Fig. 3 Dependence of the observed absorbance at 348 nm on the molar ratio r with [BSA] = 1.53 x 10^{-5} mol dm^{-3}.

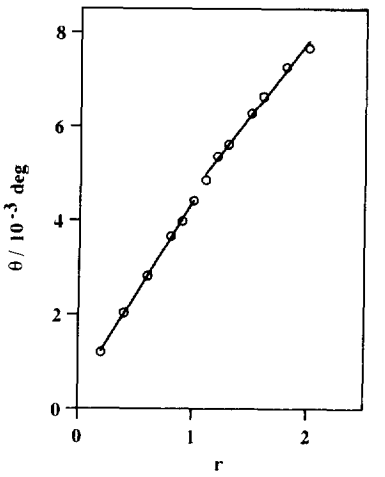


Fig. 4 Dependence of the observed ellipticities (θ) on the molar ratio r with [BSA] = 1.53 x 10^{-5} mol dm^{-3}. Values of θ were obtained from θ (peak at 334 nm) - θ (trough at 311 nm).

At all events, we can deduce that site 1, with a higher discriminating ability, also possesses a higher affinity towards the substrate than site 2 and hence uptake of 2-HT occurs predominantly at site 1 up to the ratio of r = 1, with subsequent uptake at site 2. From the enhancement of the ellipticity by *ca.* 16% in the above experiments, the ability to recognize chirality at site 2 can be estimated to be approximately 72 % of the ability at site 1, indicating a fairly good consistency with the value of 73 % obtained from the result of Fig. 4 (*vide supra*). It might be thought that (*P*)-2-HT already complexed to BSA at site 1 would affect chirality of racemic 2-HT coming close to site 2, or both 2-HT molecules complexed at site 1 and 2 would interact to each other. However, reasoning from the analysis of the crystal structure of HSA molecules in which the domains endowed with a substrate-binding site are too far apart from one another to undergo any interactions,[18] our situation concerning BSA may involve ,if any, little interaction such as a chirality control between the substrates at site 1 and 2.

Temperature-Dependence of Chirality Recognition. The molar ratio between *P* and *M* enantiomers of 2-HT incorporated into BSA manifests the ability to recognize chirality, which determines the intensities of the CD absorptions. Therefore, in order to investigate the thermodynamics of the chirality recognition in this

system, the temperature-dependence of the CD absorptions was examined for both complexes of r = 1 and 2. Upon raising the temperature within the range of 3.3 to 51.4 °C, the CD absorptions were significantly reduced for solutions of both complexes, in contrast to only a small temperature-dependence for the CD absorptions of (*P*)-[7]TH in CH_2ClCH_2Cl within the temperature range investigated here. Figure 5 illustrates the alteration in the CD spectra for the 2-HT-BSA solution of r =1. Figure 6 shows plots for the changes in the CD-absorptional intensities in the case of r = 1 and 2 at all the measured temperatures. It was confirmed that the intensity of the CD absorptions at room temperature is fully reproduced within experimental error after the solution was returned from 50 °C or 5 °C to room temperature. This fact implies that the processes caused by the temperature variation are completely reversible and no significant denaturation of BSA in an irreversible manner takes place under the present experimental conditions. This marked tendency in the CD-absorptional intensities can be explained in terms of the molecular motility of both components involved. With the rise of temperature, the thermal motion of BSA becomes gradually activated so as to weaken the configurational fixation of 2-HT molecule, resulting in the displacement of the following equilibrium to the right.

$$\text{BSA–}(P)\text{2-HT} \underset{}{\overset{K_{dia}}{\rightleftharpoons}} \text{BSA–}(M)\text{2-HT} \qquad K_{dia} = \frac{[\text{BSA–}(M)\text{2-HT}]}{[\text{BSA–}(P)\text{2-HT}]}$$

Therefore, as the alteration in the CD-absorptional intensities of the 2-HT-BSA solution of r = 1 reaches substantially a plateau below *ca.* 10 °C, a decrease in the apparent ellipticity above 10 °C may be assumed to arise from the generation of the diastereoisomeric BSA-(*M*)2-HT complex.

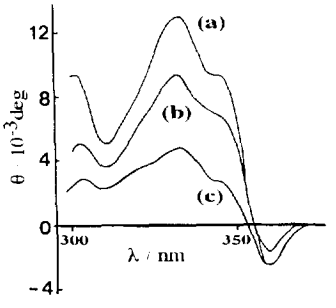


Fig. 5 Temperature-dependence of CD spectra of an aqueous 2-HT-BSA solution of r = 1 with [2-HT-BSA] = 3.06 x 10^{-5} mol dm^{-3}: (a) 6.0 °C, (b) 38.6 °C, (c) 51.4 °C.

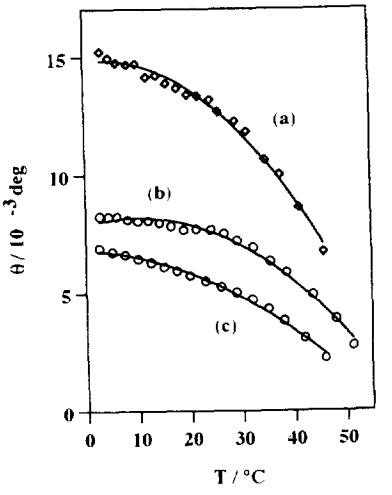


Fig. 6 Temperature-dependence of the observed ellipticities (θ) with [BSA] = 3.06 x 10^{-5} mol dm^{-3}: (a) θ_a for solution of r = 2, (b) θ_b for solution of r = 1, (c) a curve obtained from (θ_a-θ_b) at the respective temperatures. Values of θ were obtained from: θ (peak at 334 nm) - θ (trough at 311 nm).

Ellipticity for species at site 1, $[\theta]_{site\ 1}$, obtained for 2-HT-BSA solutions of r = 1 can lead to an estimation of $[\theta]_{site\ 2}$ through the relation $[\theta]_{site\ 2} = [\theta]_{site\ (1+2)} - [\theta]_{site\ 1}$, where $[\theta]_{site\ (1+2)}$ is the apparent

ellipticity measured for 2-HT-BSA solutions of r = 2 with the same BSA concentration. The curve (c) in Fig. 6 represents the plots of the values of $[\theta]_{site\ 2}$ thus obtained. In this manner the population of each diastereomeric complex can be evaluated for the two sites, respectively, to provide equilibrium constants (K_{dia}) at each temperature. Table 1 lists some selected K_{dia} values, along with the thermodynamic parameters at site 1 and 2, respectively. The occurrence of the diastereomeric complex containing *M*-enantiomer of 2-HT was found to be in a higher proportion at site 2 than at site 1. The values of ΔG^0 estimated for both sites show all positive, though small, implying that BSA-(*P*)2-HT is thermodynamically somewhat more stable than its diastereomeric isomer BSA-(*M*)2-HT.

Table 1. Equilibrium Constants and Thermodynamic Parameters at Site 1 and 2.

Site 1			Site 2		
T/K	K_{dia}	ΔG^0/kJ mol^{-1}	T/K	K_{dia}	ΔG^0/kJ mol^{-1}
283.3	0.012	10.4	283.2	0.122	5.0
294.5	0.034	8.3	293.2	0.179	4.2
302.4	0.067	6.8	302.2	0.244	3.5
311.8	0.169	4.6	311.2	0.368	2.6
321.3	0.360	2.7	319.2	0.576	1.5
ΔH^0/kJ mol^{-1}	ΔS^0/J K^{-1}mol^{-1}		ΔH^0/kJ mol^{-1}	ΔS^0/J K^{-1}mol^{-1}	
70.3	210		28.5	82.8	

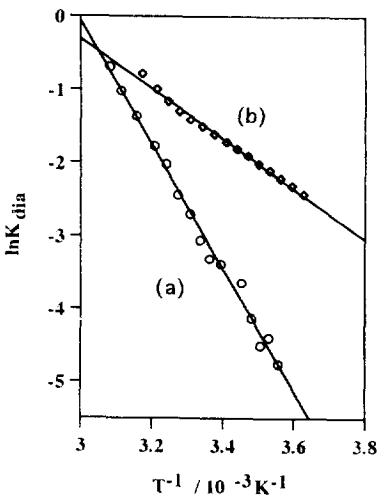


Fig. 7 The van't Hoff plots for two uptake sites of BSA: (a) site 1, (b) site 2.

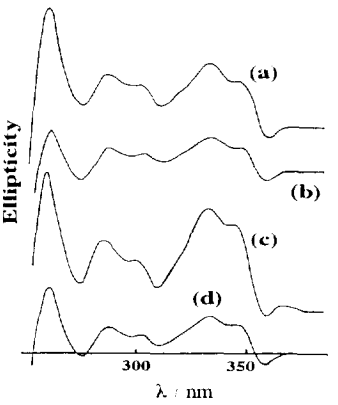


Fig. 8 The effect of heat-pretreatment of BSA for 1 hr on the CD spectra of 2-HT-BSA complex with [BSA] = 1.53 x 10^{-5} mol dm^{-3}. r = 1 solution using untreated BSA (a) and pretreated BSA (b), and r = 2 solution using untreated BSA (c) and pretreated BSA (d).

When the equilibrium constants were plotted according to the van't Hoff equation, good straight lines were obtained (Fig. 7) to give ΔH^0 and ΔS^0 values for site 1 and 2, respectively (Table 1). The ΔH^0 values imply

differing binding strengths for the BSA-(*P*)2-HT against the BSA-(*M*)2-HT species, and thus explain the difference in ability to recognize chirality of 2-HT at site 1 and 2. The larger ΔH^0 value at site 1 is in reasonable accordance with the observed behavior of higher recognition ability at site 1 (Fig.4). As for ΔS^0, the obtained values were found to be positive at both site 1 and 2, and showed much larger at site 1 than that at site 2. This trend may be explicable in terms of the sterical factors: *P*-enantiomer of 2-HT is placed under more restrained or fixed situation at the uptake sites of BSA than M-enantiomer, and moreover, the restraints subjected to 2-HT by BSA are greater at site 1 than at site 2, suggesting the greater configurational fixation for 2-HT at site 1. It has become obvious that these thermodynamic parameters, ΔG^0, ΔH^0 and ΔS^0, are effective for explaining the propensity of chirality recognition against 2-HT at each uptake site of BSA.

Pretreatment of BSA Solution with Heat. An aqueous solution of BSA was preincubated in a thermostat at 50, 60, and 70 ± 1 °C, respectively. Then, the abilities of the thus treated BSA for the substrate uptake and the chirality recognition were investigated for the 2-HT-BSA complexes of r = 1 and 2 at 25 ± 1 °C, in order to learn how site 1 and 2 change with heat-treatment. Table 2 shows the relative intensities of the UV and CD absorptions of 2-HT-BSA(pretreated) complex using one-hour pretreated BSA, in comparison with those of 2-HT-BSA(untreated) complex. Regarding the aqueous solution containing merely BSA pretreated for 1 hr, the UV-absorptional intensities are entirely unaltered up to 70 °C (measured at 278 nm) and the CD-absorptional intensities decreases to 0.98 at 60 °C, and to 0.83 at 70 °C (measured at 260 nm). In the presence of 2-HT, the 2-HT-BSA(pretreated) complex gave the unaltered intensities of UV absorptions except for a little decrease at site 2 at 70 °C, indicating almost no pronounced influence of heat pretreatment of BSA on its ability for the substrate uptake. On the other hand, the relative intensities of CD absorptions at 70 °C are significantly reduced to 0.44 for site 1 and to 0.21 for site 2, as illustrated for the complexes of r = 1 and 2 in Fig. 8.

Table 2. The Effect of Preheating of BSA on the UV- and CD-Absorptional Intensities of 2-HT-BSA.[a)]

		Site 1		Site 2	
T/°C	Time/min	UV	CD	UV	CD
50	60	0.99	1.01	1.00	0.82
60	60	1.01	0.74	0.98	0.49
70	10	1.01	0.66	0.94	0.51
70	60	1.01	0.44	0.91	0.21
70	180	0.98	0.29	1.03	0.11

a) Relative intensities against the UV and CD absorptions of 2-HT-BSA (untreated)

Table 2 also shows the effect of the pretreatment time at 70 °C on the UV- and CD-absorptional intensities of 2-HT-BSA(pretreated) complex. In this case also, prolonged heating of BSA solution does not affect the substrate uptake by BSA. In contrast, three-hour pretreatment gives a large depression of ability to recognize chirality of 2-HT, up to 0.29 for site 1 and 0.11 for site 2, though pretreatment for only 10 min already gives 0.66 and 0.51 of initial activity for site 1 and 2, respectively. A series of these facts suggest that heat pretreatment of BSA solution may bring about a small denaturation of the protein even at 70 °C, which, however, does not affect substantially the uptake ability against 2-HT , while it causes a great depression of ability to recognize chirality of enantiomers of 2-HT. This may be presumably due to the subtle conformational

deformation of BSA such as a slight change in shape or size of the substrate-binding sites. It is recognizable that such sort of deformation is somewhat larger at site 2 than at site 1.

CONCLUSIVE REMARKS

This work is the first instance proving that the inclusion phenomenon can be sufficient to cause a second-order asymmetric transformation. It was possible for us to find this transformation using BSA, by application of a new type of substrate 2-HT possessing an appropriate racemization rate and an enormous molecular ellipticity for its CD absorptions. The substrate 2-HT also allowed the investigation of the thermodynamic behavior, which provided the novel information regarding the substrate-binding sites of BSA and their ability to recognize chirality. A lowering of ability to recognize chirality can be detected by use of 2-HT for the slightly denaturated BSA produced by heating mildly. This manifests that 2-HT may act as an effective probe for assessing the ability for chiral recognition of albumins and some other proteins.

EXPERIMENTAL

Instruments. All the CD experiments were carried out with a JASCO J20A spectropolarimeter using jacketed cylindrical quartz cells of 1.0 and 0.2 cm pathlength. UV spectra were obtained on a HITACHI UV220 spectrophotometer. ^{1}H-NMR spectra were recorded on a JEOL JNM-GX270 NMR spectrometer. Sample solutions were incubated with a Yamato water bath incubator BT-25.

Materials and Procedures. Racemic tieno[3,2-e:4,5-e']di[1]benzothiophene ([5]thiaheterohelicene, [5]TH) and bisthieno[3',2':4,5]benzo[1,2-b:4,3-b']di[1]benzothiophene ([7]TH) were prepared by photocyclization of the precursor olefins.[19] (*P*)-[7]TH enantiomer was resolved from racemic [7]TH by a preparative HPLC using a chiral column according to the procedures in the literature.[14] BSA (essential fatty acid free, molecular weight 66000) was purchased from Sigma (A6003). Water used in these experiments was distilled and then passed through a Millipore Milli-QIII purification system.

[5]thiaheterohelicene-2-aldehyde.[20] [5]TH (14.36 g, 48.5 mmol) was placed in an oven-dried round bottomed flask and dissolved in 300 ml of dry THF. The flask was equipped with a dropping funnel and cooled with an ice bath. The 15 % hexane solution of n-BuLi (57.0 ml, 90.5 mmol) was added dropwise to the [5]TH solution for 30 min, and the mixture was stirred for 1 hr at room temperature. DMF (6.65 ml, 85.9 mmol) was added dropwise to the solution with cooling, and then the solution was refluxed for 1 hr. The reaction mixture was poured into the same volume of water with stirring for 40 min. The organic phase was partially evaporated under reduced pressure to give the precipitate which was separated by filtration and dissolved in 1000 ml of CH_2Cl_2. The filtrate was extracted with CH_2Cl_2. After the combined organic solution was dried over anhydrous Na_2SO_4, the solvent was removed by using a rotary evaporator. [5]TH-2-aldehyde (8.23 g, 25.4 mmol, 52.4 %) was obtained from the benzene eluent on silica-gel column chromatography. δ_H ($CDCl_3$) 10.19 (1 H, s), 8.04 (1 H, d, J 8.8), 7.99 (1 H, d, J 8.8), 7.97 (1 H, s), 8.23 (1 H, d, J 5.4), 7.9 (1 H, d, J 8.8), 7.87 (1 H, d, J 8.3) and 7.77 (1 H, d, J 5.4 Hz) ; m/z (Found: M^+ 323.9728, Calcd for $C_{17}H_8OS_3$: 323.9738).

2-hydroxymethyl[5]thiaheterohelicene (2-HT). To the solution of [5]TH-2-aldehyde (2.53 g, 7.80 mmol) in 2300 ml ethanol, $NaBH_4$ (1.18 g, 31.2 mmol) was slowly added with stirring. After the completion of the reaction was ascertained by TLC (silica-gel, CH_2Cl_2/ n-hexane (1 : 1)), the solvent was

evaporated to *ca.* 50 ml under reduced pressure. To the concentrated solution was added 100 ml of water and then the reaction mixture was shaken with CH_2Cl_2. The organic layer was dried over anhydrous Na_2SO_4, the solvent being evaporated. The residue was recrystallized from CH_3CN to yield pale yellow crystals of 2-HT (2.08 g, 6.37 mmol, 73.5 %). δ_H 8.24 (1 H,d, J 5.5), 8.15 (1 H, s), 7.94 (1 H, d, J 8.4), 7.87 (1 H, d, J 8.4), 7.80 (1 H, d, J 8.8), 7.72 (1 H, d, J 8.4), 7.64 (1 H, d, J 5.5 Hz), and 5.02 (2 H, s) ; (Found : C, 62.63 ; H, 3.16, Calcd for $C_{17}H_{10}OS_3$: C, 62.55 ; H, 3.09%); λ_{max} (EtOH)/nm 358sh (log ε /dm^3 mol^{-1} cm^{-1} 3.89), 343 (4.32), 330 (4.36), 314sh (4.08), 302 (3.91), 280 (4.36), 263.5 (4.39) and 240.5 (4.49).

The solution of 2-HT-BSA complex. The solution of 2-HT-BSA complex of r = 1 was prepared by mixing 0.1 ml of an ethanolic stock solution of 2-HT (1.53×10^{-3} mol dm^{-3}) with 5 ml of an aqueous solution of BSA (3.06×10^{-5} mol dm^{-3}), followed by adjusting the total volume to 10 ml with water to give the same concentration of 1.53×10^{-5} mol dm^{-3} for both components. The mixture showed slightly turbid, but a gentle agitation for 30 min at 25 ± 1 °C made the mixture a transparent solution. λ_{max} (1 % ethanol-water)/nm 360sh (log ε /dm^3 mol^{-1} cm^{-1} 3.94), 348 (4.25), 333.5 (4.31), 318sh (4.08), 303sh (3.96), 279 (4.77), 269sh (4.74), 259sh (4.65) 260sh (4.65), and 252sh (4.61) ; [θ] 780 deg dm^2 $dmol^{-1}$ (369 nm), -6400 (359.5), 27450 (346.5), 37390 (333), 10720 (310), 22880 (301), 28500 (286.5), 9150 (275.5), and 54640 (260).

The solution of 2-HT-BSA of r = 2 was prepared by the same procedures using another stock solution of 2-HT (3.06×10^{-3} mol dm^{-3}). The concentration of 2-HT in the solution measured was 3.06×10^{-5} mol dm^{-3}.

Ether extraction of 2-HT-BSA solution of r = 1. Fifteen ml of the 2-HT-BSA solution of r = 1 with the BSA concentration of 3.06×10^{-5} mol dm^{-3} in 1 % ethanol-water was extracted with 15 ml of diethyl ether. The ethereal layer was dried over several particles of molecular sieves (3Å) and filtrated with a membrane filter (0.45 mm). The ethereal solution thus obtained demonstrated the UV absorptions originated from 2-HT, but no CD absorptions. The aqueous layer treated under reduced pressure at room temperature in order to remove the dissolved ether gave the CD absorptions originated from BSA only. To 10 ml of this aqueous solution, 0.1 ml of ethanolic stock solution of 2-HT (3.06×10^{-3} mol dm^{-3}) was added and the mixture was agitated gently at 25 ± 1 °C for 30 min, reproducing the CD absorptions originated from the 2-HT-BSA complex.

The number of substrate-binding sites of BSA. Thirteen solutions with different molar ratio of 2-HT-BSA (r = 0.2 ~ 2.0) were prepared by the similar procedures mentioned above with keeping the BSA concentration 1.53×10^{-5} mol dm^{-3}. All the solutions were incubated for 30 min at 25 ± 1 °C with agitating gently and the UV and CD spectra of the solutions were measured. Furthermore, 2-HT-BSA mixture of r = 5 was stirred at room temperature for 30 hours. However, as the mixture still remained turbid, then the insoluble 2-HT was filtered off and the spectral data of the filtrate were obtained. Abs. at 348 nm: 0.636, and θ at 334 nm: 10.02 m deg.

Temperature dependence of CD absorptions of 2-HT-BSA complex. 2-HT-BSA solution of r = 1 or 2 with the BSA concentration of 3.06×10^{-5} mol dm^{-3} was placed in a jacketed cylindrical quartz cell equipped with a HAAKE circulator D3-G. Temperatures were read from a TOA DC microvoltmeter PM-17A connected with a copper-constantan thermocouple whose tip was placed inside the cell. The temperature was first varied from 3 °C to 52 °C, then returning to room temperature. The shapes and intensities of the CD absorption peaks were fully reproduced at the same temperature going back and forth.

Migration of 2-HT from site 2 to site 1 of BSA. To 10 ml of 2-HT-BSA solution of r = 2 with the BSA concentration of 3.06×10^{-5} mol dm^{-3} were added 5 ml of the aqueous BSA solution of 6.12×10^{-5}

mol dm^{-3} and 0.1 ml of ethanol. The total volume of the mixture was adjusted to 20 ml with water, and then the solution was incubated with stirring mildly at 25 ± 1 °C for about 24 hours.

Heat pretreatment of BSA solution. BSA solutions of 1.53×10^{-5} mol dm^{-3} were incubated in a thermostat at 50, 60, and 70 ± 1 °C, respectively. Samples for measuring the spectral data were made up at room temperature by the above mentioned procedures using this preincubated BSA solution.

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