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Conformational Changes of Human Serum Albumin by Binding of Small Molecules

HIROSHI UCHIDA[1a)] and MANABU HANANO[1)]

Faculty of Pharmaceutical Sciences, University of Tokyo[1)]

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A fluorescent probe specific for sulfhydryl group was synthesized. Conformational change in HSA was observed by fluorescence spectral shift on binding. From the experimental evidences of urea denaturation and fluorometric pH titration, phenylbutazone or secobarbital binding was concluded to induce the conformational change of HSA from the native state to a more expanded form.

It has been noted that a binding of small molecule to macromolecule may induce conformational changes of macromolecules which take place in such a biological phenomenon as an allosteric effect, an exposure of successive binding site, or a denaturation. The observation of a local structure change at definitive region may be required for the reversible conformational changes.

HSA[2)] has an ability to bind various kinds of small molecules such as fatty acids, dyes and drugs. A nonspecificity of HSA for the binding might be attributed to both its structural flexibility and its hydrophobicity. HSA is so flexible that it would be possible to detect conformational changes resulting from molecular binding. Although there, in HSA, are 17 sulfhydryl groups which are exchangeable between thiol and disulfide type, HSA contains one reactive sulfhydryl group around a neutral pH.[3)]

It has been shown in HSA by Ohkubo[4)] that the reactive sulfhydryl group is located at the border between a polar helical segment and a hydrophobic region, and that lone tryptophan residue is located in a neighborhood of such a reactive sulfhydryl group on a surface. From observations that tryptophan fluorescence was quenched by binding of ion or small molecule and that the more hydrophobic molecule has the higher affinity to HSA, the hydrophobic segment containing tryptophan residue has been proposed for the binding site in HSA. Accordingly, one can assume that fluorescence properties of probe bounded to sulfhydryl group will be altered by an interaction of small molecule with such a binding site in HSA.

In this study, thus, it is aimed to label the reactive sulfhydryl group by a fluorescent probe and to detect conformational changes induced with drug binding around the labelled region.

Experimental

Materials——HSA (Frac. V Powder) and HMA (crystallized) were purchased from Miles Laboratories, and were used without further purification. Their concentrations, were determined on the basis of weight of dried sample. The assumed molecular weight was 66000. Phenylbutazone was kindly supplied by Mr. Fukumuro (Hospital of University of Tokyo). Other chemicals were obtained from many distributors as reagent grade. All water used was deionized and distilled. Unless otherwise indicated proteins were dissolved in 0.1 M Tris-HCl (pH 7.4).

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1) Location: *Hongo, Bunkyo-ku, Tokyo*; *a*) Present address: *Kyorin Chemical Laboratories, Ukima, Kita-ku, Tokyo.*
2) The following abbreviations used are: HSA, human serum albumin; HMA, human mercaptoalbumin; DPA, *p*-dansylamino-phenylmercuric acetate; PCMB, *p*-chloromercuribenzoic acid.
3) E. Katchalski, G.S. Benjamin, and V. Gross, *J. Am. Chem. Soc.*, **79**, 4096 (1957).
4) A. Ohkubo, *J. Biochem.*, **65**, 879 (1969).

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Synthesis of *p*-Dansylamino-phenylmercuric Acetate (DPM)——In a typical preparation of DPM, 0.6 g (0.022 moles) of dansyl chloride (Seikagaku Fine Biochemicals) was dissolved in 40 ml benzene, and followed to be filtered to remove a small amount of insoluble materials. To this yellow solution, 0.8 g (0.023 moles) of *p*-aminophenylmercuric acetate was added and moreover 10 ml pyridine was poured to make reaction complete. The above suspension became to red solution with red precipitates as reaction proceeded by vigorous stirring at room temperature for 1 hour, at which time an end of reaction was indicated by disappearance of white *p*-amino-phenylmercuric acetate powder. The red powder was separated through filtration and moreover red powder was obtained by adding an aliquot of benzene to the filtrate. The combined red powder was washed with benzene a few times and dried *in vacuo*. *Anal.* Calcd. for $C_{20}H_{20}N_2$: C, 41.06; H, 3.45; N, 4.79. Found: C, 40.20; H, 3.26; N, 4.78. The small discrepancy between found and calculated values of an elementary analysis was perhaps attributed to the difficulty of purification.

Modification of HSA with DPM——Pyridine solution of DPM was added stepwisely into HSA solution (pyridine/tris buffer$<$1%) at room temperature. The reaction mixture was stood for a few hours, filtered and then dialyzed extensively against tris buffer, which treatments were carried out in a cold room below 4°. A measurement of pH and ultraviolet absorption was made with TOA, MH-5A, pH meter and Hitachi, 124, spectrophotometer respectively. Fluorescence was measured with Hitachi, 203 fluorescence spectrometer equipped with mercury lamp. All emission spectra represented in this study were not corrected.

Result

Both emission spectra of free and a bound DPM were given in Fig. 1. The following two important features were derived from these spectra: an increase in fluoresence intensity and marked wavelength shift to shorter wavelength on binding. Since various hydrophobic fluorescent probes have been known to show the stronger fluorescence and the more pronounced shift to shorter wavelength in the less polar solvent, a measurement of DPM fluorescence spectrum was carried out in a solution with various compositions of pyridine and tris buffer in order to know a nature of DPM. This result is represented in Fig. 2. As these spectra were obtained at the same condition except the composition of solvent, one can directly compare their fluorescence intensities and peak positions each others. It is apparent that DPM showes the more enhanced fluorescence and the larger shift to shorter wavelength in the less polar solution as other fluorescent probes do. This provides the possibility to be able

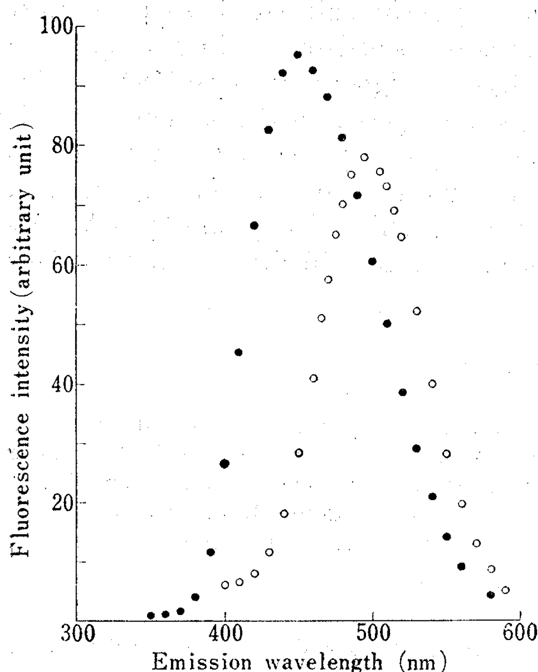

Fig. 1. Fluorescence Spectrum of DPM or DPM–HSA

○: 40 μM DPM ●: 7.6 μM DPM–HSA
Excitation was made at 310 nm.

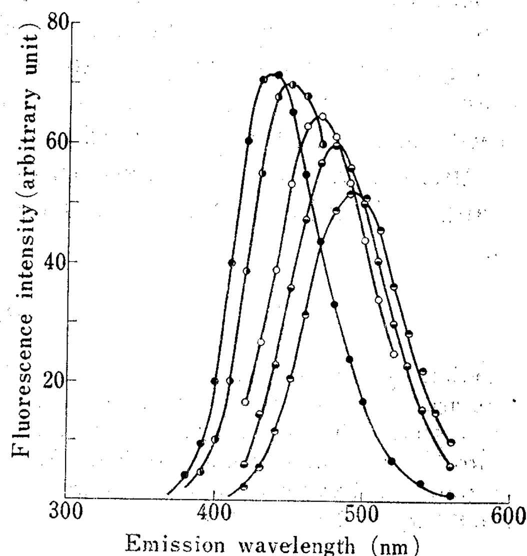

Fig. 2. Fluorescence Spectra of DPM in Various Pyridine–Water Mixtures

●: 100% pyridine ◐: 90% pyridine
○: 70% pyridine ◒: 30% pyridine
◓: 10% pyridine
Excitation was made at 310 nm. DPM concentration of each solution was 13.3 μM.

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to use DPM as the hydrophobic probe. Following the observation of D.A. Deranleau and H. Neurath[5] that a big shift in emission spectrum occured toward the shorter wavelength when dansylated L-tryptophan ethyl ester was bound to chymotrypsin or chymotrypsinogen, a large shift (about 45 nm), as shown in Fig. 1 will suggest that DPM is located at the more hydrophobic environment in HSA rather than in tris buffer.

In the combination of dansylated L-tryptophan ethyl ester with chymotrypsin or chymotrypsinogen, identical emission spectra were observed when the above probe was either covalently bounded or when the ligand was bound to the protein in the equilibrium sense. It was, thus, subjected to investigate either DPM was covalently bounded to HSA or physically bound on a surface of HSA. Physically bound molecule was reasonably considered to be so easily transfered toward organic phase that amount of DPM extracted in chloroform phase should increase with the increase of volume of chloroform linearly, until all DPM in the buffer phase was extracted, when a constant volume of an aqueous mixture of HSA and DPM was shaken with a various volume of chloroform. Relative amounts of DPM in chloroform and buffer phase were given with a relative fluorescence intensity in chloroform phase in Fig. 3. It is so difficult to gain an absolute value because of its low solubility that the relative amounts were plotted instead of the absolute ones in Fig. 3. The relative value will be enough for a check of DPM partition between two phases. Both the amount of DPM transfered to chloroform phase and of DPM remaining in buffer phase were lebelled off, increasing volume of chloroform. This evidence might suggest the possibility that a part of DPM in the mixture was covalently bound to HSA. The another important evidence is that the same fluorescence intensity was observed in chloroform phase, regardless of the presence of phenylbutazone in

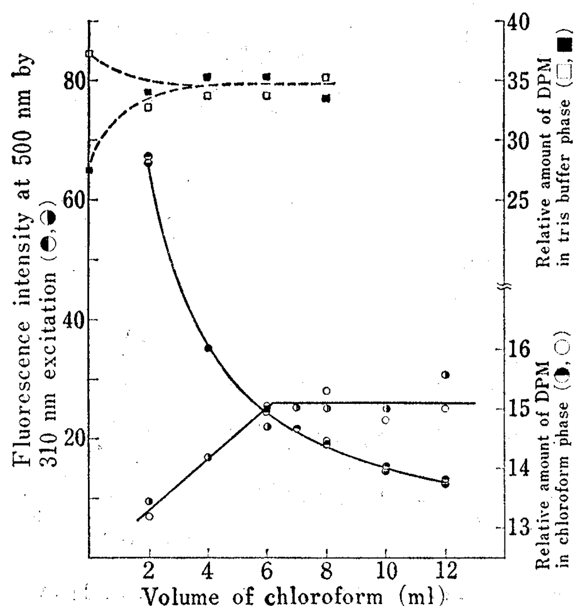

Fig. 3. Effect of Chloroform Extraction on Fluorescence and Relative Amount of Chloroform and Aqueous Phase

Relative intensity of chloroform phase at 500 nm when excited at 310 nm, relative amount of DPM in Tris-buffer phase and chloroform phase after chloroform extraction from 3 ml Tris-buffer solution containing DPM (3.88 μM) were shown as ◓, □, and ◑, respectively. The corresponding values from Tris-buffer solution containing DPM (3.88 μM), HSA (1.65 μM) and phenylbutazone (21.4 mM) were demonstrated as ◒, ■, and ○, respectively.

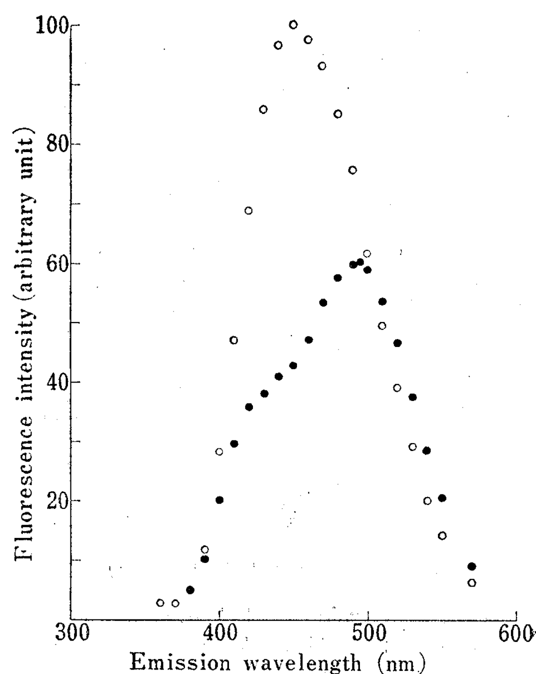

Fig. 4. Effect of Phenylbutazone on Fluorescence Spectrum of DPM–HSA

○: DPM–HSA (1.13 μM), ●: DPM–HSA (1.13 μM) and phenylbutazone (0.13 mM)
Excitation was made at 310 nm.

5) D.A. Deranleau and H. Neurath, *Biochem.*, 5, 1413 (1966).

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the aqueous solution. If DPM was only physically bound on HSA, a coexistence of phenylbutazone would enhance the fluorescence intensity in chloroform phase due to the displacement of the adsorbed DPM, being shown for phenylbutazone to be able to displace a dansylglycine adsorbed on HSA surface.[6] This evidence also will provide the possibility of covalent bond between DPM and HSA.

There are many studies about the reactivity between organic mercurials and sulfhydryl group of proteins. DPM was expected to react with sulfhydryl group in HSA in a sense of its structure. For the estimation of the reactivity of DPM with HSA, the emission spectral change was used at pH 7.4, at which pH HMA had been ascertained to have a lone reactive sulfhydryl group per molecule. It was obvious from the crossover point of two straight lines that DPM could react with HMA at a molar ratio of one to one. In order to obtain the more assurance of the reactivity, the unmodified or modified HMA was titrated with PCMB, which had been known a specific sulfhydryl reagent.[7] As expected, PCMB could react with unmodified HMA, but not with the modified HMA. From these results, DMP was concluded to be covalently bounded to the lone reactive sulfhydryl group in HSA.

The fluorescence spectrum of DPM attached to HSA was markedly changed with an addition of phenylbutazone. The emission spectra shown in Fig. 4 was normalized to 1.0 at the wavelength (450 nm) of emission maximum with DPM attached to HSA. It is particularly

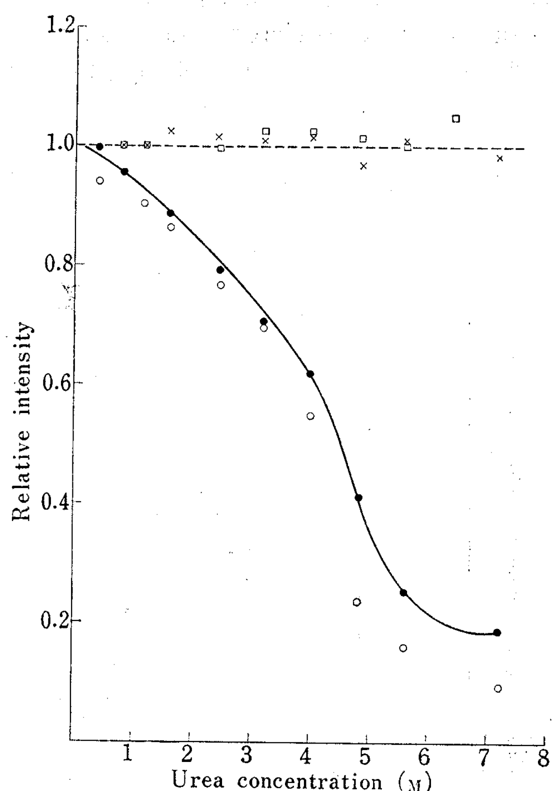

Fig. 5. Intrinsic or DPM Fluorescences of HSA and DPM–HSA as a Function of Urea Concentration

□: $F_{275\to350}$, HSA (1 mg/ml); ×: $F_{275\to350}$, DPM–HSA (1.2 mg/ml); ○: $F_{275\to450}$, DPM–HSA (1.2 mg/ml); ●: $F_{310\to450}$, DPM–HSA (1.2 mg/ml)
$F_{x\to y}$ means relative fluorescence intensity at y nm when excited at x nm.

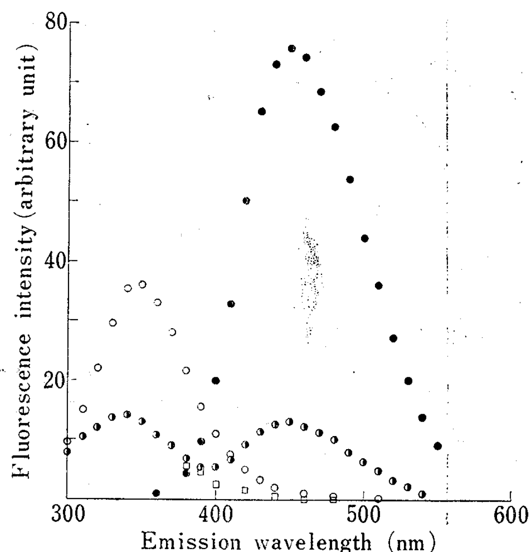

Fig. 6. Fluorescence Spectrum of HSA or DPM–HSA

Fluorescence spectrum of HSA (1.0 mg/ml) when excited at 275 nm and 310 nm were shown as ○ and □, respectively.
The corresponding spectra of DPM–HSA (1.2 mg/ml) were shown as ◑ and ●, respectively.

6) C.F. Chignell, *Mol. Pharmacol.*, **5**, 244 (1969).
7) P.D. Boyer, *J. Am. Chem. Soc.*, **76**, 4331 (1954).

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interesting to find out that the emission spectrum with phenylbutazone has a peak (495 nm) based on free DPM and a shoulder of the bounuded DPM in HSA, as shown in Fig. 1.

The similar spectral change was observed on the addition of urea to the modified HSA solution. Comparing the effect of urea with that of phenylbutazone, urea affected the emission spectrum of the bounded DPM more profoundly. Since an urea denaturation had been extensively studied on HSA, measurements of intrinsic and DPM fluorescence were conducted at the various molar concentrations of urea, in order to take an information about structural perturbation in HSA on phenylbutazone binding. The measurement was carried out at 2 or 3 hours after the solution had been prepared, because the intrinsic fluorescence of bovine serum albumin was reported to vary with time in the presence of urea.[8] Although the intrinsic fluorescence of bovine albumin has been known to be diminished by increasing urea concentration, that of HSA was practically constant over the same urea concentration region. In Fig. 5, such an evidence was given with the results of the bound DPM obtained at tryptophan excitation wavelength (275 nm) and at DPM maximun excitation wavelength (310 nm). The intrinsic fluorescence of the modified HSA was also not altered within an experimental error as the unmodified HSA. On the other hand, DPM fluorescence in HSA was sensitive to urea concentration either the excitation was made at 275 nm or 310 nm. The measurement of a specific viscosity[9] or a specific rotation[10] revealed that HSA might been extended form in an urea solution of higher than 3 or 4 molar concentration, because each parameter indicated a critical change at such an urea concentration, suggesting an molecular expansion. The fluorescence intensity of DPM in HSA also decreased sharply at the same urea concentration, as shown in Fig. 5. This good correspondence will suggest that the DPM fluorescence spectral change with urea reflects any gross structure perturbation, including the structural modification around the reactive sulfhydryl group.

The unmodified HSA showed only tryptophan fluorescence (350 nm), however, the modified HSA clearly showed two emission peaks attributed by tryptophan residue and the bound DPM when excited at 275 nm. The tryptophan fluorescence was indeed diminished in the modified HSA, in Fig. 6. Taking a consideration either an energy transfer from tryptophan residue to the bounded DPM takes place or the enviromental change around tryptophan residue is induced during a process of chemical modification with DPM, the above decrease of tryptophan fluorescence would reasonably explained. It is very difficult to interpret the intrinsic fluorescence mechanically, because the detail informations about the local environment around tryptophan residue are still lack, even though the tryptophan segment was isolated from HSA by an extensive work of Klotz, *et al.*[11]

In order to gain insight into the nature or mechanism of conformational change with phenylbutazone or urea, a fluorometric pH titration was performed with the free or the bounded DPM, because conformational change in HSA had been well studied over a wide pH range. The fluorescence spectrum of the bounded DPM at low pH was similar to that of free DPM, that is, the emission peak shifted from 450 nm at pH 7 to 495 nm at pH 2. The fluorescence intensity was remarkably diminished at low pH below 4. The fluorescence intensity at 450 nm significantly decreased around pH 4. On the other hand, a transitional pH of free DPM was about 6.5. It is interesting to compare this pH dependence with results on conformational change induced by hydrogen ion binding. Bovine and human serum albumins have been known to undergo a marked structural change around pH 4. Hori and Aoki,[12] studying an electrophoretic change occurring in HSA at 0.1 of ionic strength of acetate buffer, showed a

8) N.A. Attallah and G.F. Lata, *Biochim. Biophys. Acta*, **168**, 321 (1968).
9) H. Neurath and A.M. Saum, *J. Biol. Chem.*, **128**, 347 (1939).
10) P. Callagham and N.H. Martin, *Biochem. J.*, **83**, 144 (1962).
11) J.B. Swaney and I.M. Klotz, *Biochem.*, **9**, 2570 (1970).
15) C.F. Chignell and D.K. Starkweather, *Pharmacol.*, **5**, 235 (1971).

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percent of native state (N) in this pH region. A form having a faster rate of migration (F) predominant at pH region below 4 is characteristic of being extended structure. This transitional pH from N to F form coincided well with that of the bounded DPM fluorescence, as seen in Fig. 7, where a percent of native state (date of Hori, *et al.*[12]) was given at the right hand ordinate for a comparison. Consequently, HSA might be transformed from native state to more extended form on phenylbutazone binding, even not so serious as urea or acid denaturation, being based on the shift and the magnitude of the spectral intensity in each case.

The fluorescence intensity was measured at various concentrations of phenylbutazone. Following a linear form of Langmuir isotherm, a binding constant was calculated to be $3.0\times10^5\,\text{M}^{-1}$ by a least-square method under the assumption that HSA had only one binding site for phenylbutazone. On this calculation, concentration of HSA, that is, that of the modified sulfhydryl group was calibrated by a factor of 0.67, since HSA had been known to be consist of two forms; one with one sulfhydryl group per molecule (two-thirds) and the another with no sulfhydryl group.[13] By ultrafiltration technique, phenylbutazone was found to bind to a single site on HSA with an affinity constant of $1.17\times10^5\,\text{M}^{-1}$.[14] Chignell, *et al.*[15] found by an equilibrium dialysis method that a dialyzed crystalline HSA had one binding site of high affinity constant ($1\times10^5\,\text{M}^{-1}$) and two other with a lower affinity, and the defatted HSA contained one binding site of a higher binding constant ($10\times10^5\,\text{M}^{-1}$) and the others with a lower affinity for phenylbutazone. The value obtained in this study can be well comparable with values reported in other investigations.

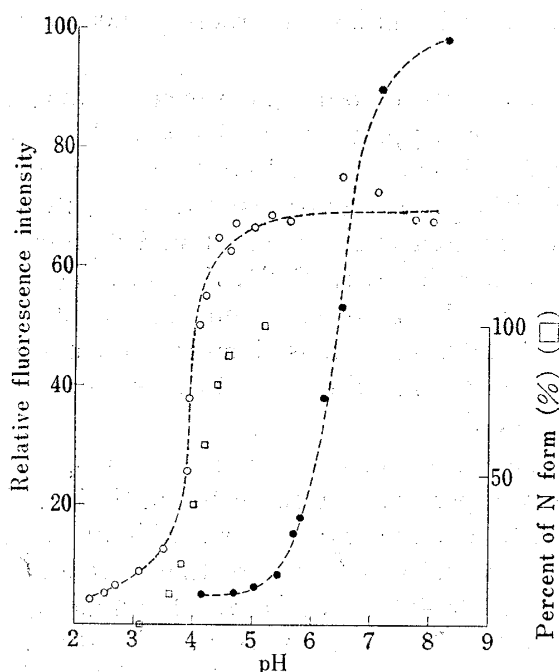

Fig. 7. Fluorescence Intensity of DPM or DPM–HSA as a Function of pH

DPM (●) and DPM–HSA (○) concentrations were 4.14 μM and 12.1 μM respectively. Tartarate buffer (pH 2.25—4.5), acetate buffer (pH 3.9—5.6), phosphate buffer (pH 6.5—8.0).

Percent of N form in HSA was demonstrated from work of J. Hori and K. Aoki.[12]

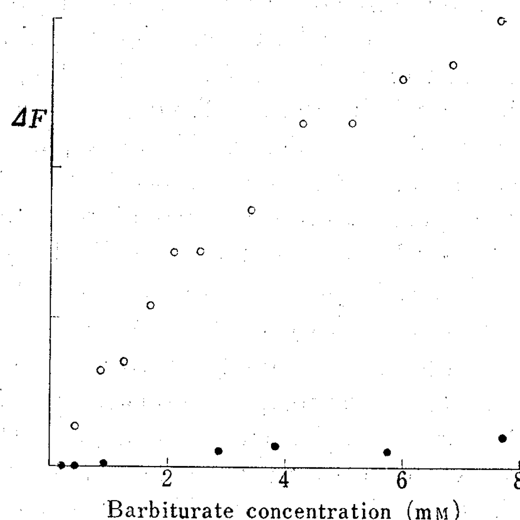

Fig. 8. Fluorescence of DPM–HSA as a Function of Barbiturate Concentration

secobarbital (○), barbital (●)

Emission intensity was measured at 445 nm at 27°, 0.1 M Tris-HCl buffer (pH 7.4).

12) J. Hori and K. Aoki, *Arch. Biochem. Biophys.*, **88**, 232 (1960).
13) Y. Gabr, M. Soliman, S. Dawoud, and E.S. Amin, *Arzneimittelforsch.*, **21**, 574 (1971).
14) H.M. Solomon, J.J. Schrogie, and D. Williams, *Biochem. Pharmacol.*, **17**, 143 (1968).

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Using the same method, the binding of barbital or secobarbital was studied. Secobarbital decreased the fluorescence intensity at 450 nm but barbital could not affect its intensity practically as shown in Fig. 8. This result is reasonable on the view that the more hydrophobic molecule is easily bound to serum albumin in a series of an analogeous derivatives. With regard to secobarbital, the similar spectral change was observed as phenylbutazone. Although the fluorescence intensity did show no observable change with barbital, it apparently decreased with barbital in the presence of zinc. A coexistence of copper ion did not so much change the DPM fluorescence intensity even the barbital concentration was high. In order to gain informations about the binding site for zinc or copper, the intrinsic fluorescence was measured with metal ion at 350 nm. As shown in Fig. 9, zinc ion did not affect the fluorescence intensity, but copper ion could quench it as similarly as the combination of copper ion with tryptophan or bovine serum albumin as studied by C.K.Luk.[16] The almost same emission specta were obtained irrespective of the existence of zinc ion, but copper ion did quench the fluorescence of the bound DPM. From the above facts, it would be considered that zinc ion bound to the segment with a long distance from tryptophan residue and DPM, and copper ion bound to or near tryptophan residue in addition to NH_2-terminal peptide which had been demonstrated as a primary binding site for copper ion.[17] It has not yet known whether no spectral change observed with barbital in the presence of copper ion will result from the gross structural change in HSA in spite of binding of barbital.

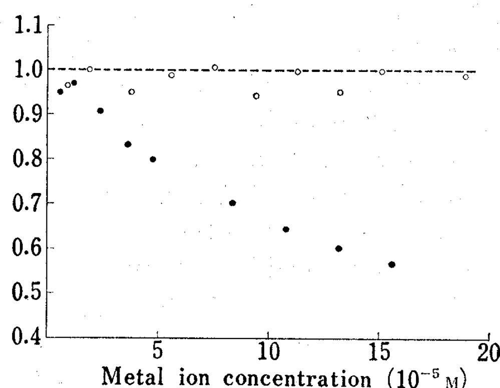

Fig. 9. Effect of Zinc or Copper Ion on Intrinsic Fluorescence of HSA

zinc ion (○); copper ion (●) The measurement was carried out at 350 nm when excited at 275 nm. HSA concentration was 1.5 mg/ml.

Discussion

A hydrophobic fluorescent probe is characterized by an enhanced intensity and a marked shift to shorter wavelength in the less polar solvent. 1-anilino-naphthalene-8-sulfonate[18] or 2-*p*-toluidinylnaphthalene-6-sulfonate[19] is almost nonfluorescent in water, but the fluorescence intensity increases and emission peak shifts to shorter wavelength with increasing carbon chain of primary aliphatic alcohol. The fluorescence of a tetracycline[20a] or a chelated chlortetracycline[20b] also is polarity dependent, being higher in the less polar solvent. Since DPM showed the same trend, it seemed one of hydrophobic fluorescent probes.

A complete primary structure of HSA has not been revealed. Amino acid sequence in the neighborhood of the single tryptophan residue has, however, been recently established as Lys–Ala–Try–Ala–Val–Ala–Arg by Swaney and Klotz.[11] This heptapeptide would be exposed to the solvent, because of charged terminal residues. It has to be pointed out that the sequence around tryptophan residue is composed of apolar residues, which is probably the binding site for small molecule, since the tryptophan perturbation was observed with cortisol or testosterone in ultraviolet region of difference spectra, and the intrinsic fluorescence quenching was recorded with cortisol.[21]

16) C.K. Luk, *Biopolymer*, **10**, 1229 (1971).
17) T. Peters, Jr. and F.A. Blumenstock, *J. Biol. Chem.*, **242**, 1574 (1967).
18) L. Stryer, *J. Mol. Biol.*, 13, 482 (1965).
19) W.O. McClure and G.M. Edelman, *Biochem.*, **5**, 1908 (1966).
20) *a*) A.H. Caswell and J.D. Hutchison, *Biochem. Biophys. Res. Commun.*, **42**, 43 (1971); *b*) Unpublished observation.
21) M.T. Ryan and G. Gibbs, *Arch. Biochem. Biophys.*, **136**, 65 (1970).

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Although HSA contains 17 sulfhydryl groups per molecule, there is the single reactive sulfhydryl group which has an ability to react with mercuric ion, organic mercuric ion, cysteine or other substances, over a neutral pH region. Amino acid sequence of cystein-containing peptide derived from HSA was shown by Witter and Tuppy[22] as follows; Leu–Gln–Asp–Glu–Gln–Glu–Cys–Pro–Phe. This peptide is consist of polar amino acid residues in particular. By the study of solvent perturbation difference spectra of unmodified, cysteinyl and glutathionyl HSA, the reactive sulfhydryl group was unvailed to be located near the tryptophan residue.[4]

Chignell[6] appreciated that the binding site for phenylbutazone was a hydrophobic region in HSA from the observations that phenylbutazone could displace dansylglycine, a hydrophobic fluorescent probe; the red shift of ultraviolet spectrum of phenyl group in phenylbutazone occurred on binding and the introduction of hydrophillic groups into phenylbutazone reduced the magnitude of optical activity induced with binding. Consequently, if phenylbutazone was bound to the hydrophobic segment around the tryptophan residue, the fluorescence of DPM bounded at sulfhydryl group would be expected to be altered by phenylbutazone binding on the view of the closeness of distance between the bound DPM and the binding site. This was confirmed in this study.

Phenylbutazone binding did not only reduce the fluorescence intensity at 450 nm, but also produced a new peak corresponding to that of free DPM. Although conformational changes of proteins have been thought to take place during their molecular interaction with small molecule, their local structure changes at the definitive portion have not clearly observed. The spectral change in the interaction of phenylbutazone with HSA would provide such an example of local structure change induced by molecular interaction.

Since the emission spectrum changes on phenylbutazone binding were similar to those induced at high urea concentration or at low pH, it was initiated to investigate closely their effects on both tryptophan and DPM fluorescences. The intrinsic fluorescence of bovine serum albumin was reported to decrease with the increase of urea concentration, but that of HSA was not reduced by urea denaturation. These evidences are attributed to the facts that at least one of two tryptophan residue in bovine serum albumin is buried in the interior of the protein, and the lone tryptophan residue of HSA is exposed on the surface as found by solvent perturbation technique.[4] In spite of insensitivity of the intrinsic fluorescence to urea, the DPM fluorescence intensity changed with urea concentration, which dependence was similar with results obtained by viscosity[9] or optical activity[10] measurement. These results probably supported the fact that tryptophan residue was already exposed to the solvent and the bounded DPM was surrounded with hydrophobic segment in the native state, being suggested by spectral shift. The resemblance between the dependences of DPM fluorescence and that of other physical parameters on urea concentration will indicate that the emission spectrum change obtained in urea denaturation reflects the structural change like a molecular expansion.

By a study of the effect of pH on the viscosity and optical rotation, it was shown that an essentially isotropic expansion took place in acid solution, which expansion occurred through an intermediate expandable form disignated with F.[23] This N-F transition has been postulated to involve the opening of a crevice[24] or the separation of intramolecular substructure.[25] Following the results that the DPM fluorescence spectrum shifted to longer wavelength and diminished at pH region below 4, and the transitional pH of the fluorescence intensity closely corresponded to that of N-F transition, the buried DPM in HSA was considered to become

22) A. Witter and H. Tuppy, *Biochim. Biophys. Acta.*, **45**, 429 (1960).
23) J.T. Yang and J.F. Foster, *J. Am. Chem. Soc.*, **76**, 1588 (1954).
24) T.T. Herskovits and M. Laskowski Jr., *J. Biol. Chem.*, **237**, 2481 (1962).
25) J.F. Foster, "in The Plasma Proteins," F.W. Putnam ed. New York N,Y., Academic Press.

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exposed to the solvent during N-F transition. The tryptophan fluorescence change was also expected in the process of N-F transition due to the closeness of tryptophan residue to cystein residue. The intrinsic emission spectrum of HSA at pH 2.77 was shifted to the shorter wavelength, compared with the spectrum at pH 5.42.[26] The fluorescence intensity of the tryptophan residue changed critically around pH 4, with that of tyrosine. Although both intensities of tryptophan and of the bounded DPM change strikingly around pH 4, their shifts are reverse. If spectral shift reflects the environmental change around moieties, the above facts suggest that tryptophan residue was converted into a more hydrophobic environment, and DPM was exposed to the solvent during N-F transition. This would suggest the molecular flexibility around the binding site in HSA. From the above facts and considerations, it will be reasonably concluded that HSA is transformed to a more expanded conformation by phenylbutazone binding.

The various kinds of segments have been proposed for the binding site of a metal ion in serum albumin. A primary binding site for copper ion is known to involve NH_2-terminal amino group, two intervening peptide nitrogens and an imidazole nitrogen of a histidine residue.[15] As another portion sulfhydryl group was also proposed from the consistence of both the absorption maximum of copper-bovine serum albumin complex and that of a copper mercaptide.[27] According to the polarographic study of C. Tanford,[28] copper ion has a higher affinity to HSA than zinc ion, and both ions bind to the imidazole in HSA. These evidences would probably suggest that copper ion could induce any structural change more severely than zinc ion, which was indicated in the marked change of DPM fluorescence with copper ion. With regard to the effect of zinc ion on barbital binding, the following three mechanisms could be considered; (1) the change of molecular hydrophobicity of barbital by chelation, (2) structural change of HSA-zinc complex which lead to an exposure of the binding site for barbital, or (3) a ternary complex formation among three components. Any conclusion could not yet be derived on the correct mechanism working in this system.

Acknowledgement We wish to thank Dr. M. Hirobe for advices with the synthesis of DPM. This work was supported in part by a grant from Minister of Education of Japan.

26) R.F. Chen, *Biochim. Biophys. Acta*, **120**, 169 (1966).
27) I.M. Klotz, J.M. Urquhart, and H.F. Fiess, *J. Am. Chem. Soc.*, **74**, 5537 (1952).
28) C. Tanford, *J. Am. Chem. Soc.*, **74**, 211 (1952).

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