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Interaction of Several Drugs with Tyr 411 Anthraniloyl Human Serum Albumin

HIROKO SAKAMOTO,* IZUMI NAGATA, MARIMO KOIKE, and MASACHIKA IRIE

Hoshi College of Pharmacy, 2–4–41 Ebara, Shinagawa-ku, Tokyo 142, Japan

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In order to establish the contribution of Tyr 411 of human serum albumin (HSA) to specific drug binding, the effect of drug binding on the fluorescence spectrum of HSA in which Tyr 411 was modified with *p*-nitrophenyl anthranilate (ANT-HSA) was studied by using various drugs (diazepam, chlordiazepoxide, flufenamic acid, indomethacin, azapropazone, phenylbutazone, warfarin, salicylic acid, ibuprofen, L-tryptophan, tolbutamide and sulfadimethoxine). Among these drugs, indomethacin and flufenamic acid strongly quenched the fluorescence of the anthraniloyl group of ANT-HSA at a molar ratio of 1.0, indicating that these drugs bind near the Tyr 411 residue.

Keywords——human serum albumin; drug binding site; anthraniloyl HSA; Tyr 411 residue; fluorometry; anti-inflammatory drug; benzodiazepine; L-tryptophan; tolbutamide; sulfadimethoxine

Many investigations have been reported on the binding of various drugs with human serum albumin (HSA), and the presence of several binding sites on the surface of HSA has been proposed by many investigators.[1-10] In order to determine the locations of these binding sites on HSA, studies on the interaction of various drugs with chemically modified HSA's or on the inhibition by various drugs of HSA modification with various modifier reagents have been carried out. Fehske and co-workers[11,12] studied the effect of chemical modification of tyrosine residues with tetranitromethane on the binding of diazepam (DAP). They showed that binding of DAP is greatly reduced by the modification of a few reactive tyrosine residues out of the 18 tyrosine residues. Furthermore, Means and his colleagues[13-15] reported that DAP and several drugs inhibited the reaction of *p*-nitrophenylacetate with the Tyr 411 residue of HSA,[16] but warfarin (WA) did not affect this modification. The same conclusion was also reached independently on the basis of similar experiments by Ozeki *et al.*[17] and Kurono *et al.*[18,19] Therefore, Tyr 411 is considered to be located within the primary binding site of DAP.

In a previous paper, in order to determine the role of the Trp 214 residue of HSA, we reported the effect of the modification of Trp 214 with *N*-bromosuccinimide (NBS) on the bindings of chlordiazepoxide (CDO), WA and phenylbutazone (PB).[20] In this work, in order to study the contribution of the Tyr 411 residue to the specific drug binding sites of HSA, the effect of drug binding on the fluorescence spectrum of HSA in which Tyr 411 was specifically modified by *p*-nitrophenyl anthranilate (NPA)[21] was studied using DAP, CDO, flufenamic acid (FA), indomethacin (IM), azapropazone (APz), PB, WA, salicylic acid, ibuprofen (IP), L-tryptophan (TP), tolbutamide (TB) and sulfadimethoxine (SDM).

Experimental

Materials——HSA obtained from Sigma Chem. Co. (crystallized) was defatted and fractionated as described in

the previous paper.[20] NPA was purchased from Sigma Chem. Co. DAP, CDO and TB were obtained from Yamanouchi Pharm. Co., Ltd., FA from Sankyo Co., IM from Japan Merck-Banyu Co., APz from Nippon Chemiphar Co., Ltd., IP from Kaken Kagaku Co. PB, WA, TP, sodium salicylate (SA) and SDM were purchased from commercial suppliers.

NPA Modification[21]——The anthraniloyl Tyr 411-HSA (ANT-HSA) was prepared by the addition of 1.3 molar eq of NPA to 18.8 μM HSA in 50 mM phosphate buffer (pH 8.0). The reaction was allowed to proceed until the release of *ca.* 1 molar eq of *p*-nitrophenol at 25 °C (7—14 h), after which the reaction mixture was dialyzed against 50 mM phosphate buffer (pH 7.0) to remove the *p*-nitrophenol produced and the excess reagent. The extent of modification was estimated from the molar extinction coefficient of ANT-HSA at 350 nm (taken as 6300 $M^{-1}cm^{-1}$).

Circular Dichroism (CD) Spectra——CD spectra were measured with a JASCO J-40 spectropolarimeter at room temperature in a cell of 0.2 cm light path for the wavelength region of 200—250 nm. The protein concentrations used were 2.0 μM.

Sodium Dodecyl Sulfate (SDS)-Slab Electrophoresis——To check the formation of aggregates or cross-linked products, SDS-slab electrophoresis of ANT-HSA was performed according to the procedure of Laemmli.[22]

Binding Studies——(a) Difference Fluorescence Spectrum: The fluorescence spectrum of ANT-HSA was determined by using an excitation wavelength of 360 nm, and the fluorescence emission at 425 nm quenched by addition of various amounts of drugs was measured at pH 7.0 (50 mM phosphate buffer) and 20 °C with a Hitachi 650-10S fluorescence spectrophotometer. The percentage of quenching was calculated from the fluorescence of the control protein solution to which an equal volume of buffer without the drug was added. The protein concentrations were 2.2 μM. The difference fluorescence spectrum due to the quenching of tryptophan residue was also measured at 350 nm with excitation at 295 nm.

(b) Difference Ultraviolet (UV) Absorption Spectrum: Difference UV spectra of ANT-HSA induced by addition of various amounts of drugs were measured at pH 7.0 and 20 °C with a Shimadzu UV 200S spectrophotometer using tandem cells. The final protein concentrations were 18.8 μM.

Results

Preparation of ANT-HSA

ANT-HSA showed almost exactly the same extinction coefficient at 350 nm as that reported by Hagag *et al.*[21] From the CD spectrum of ANT-HSA in the short wavelength region (200—250 nm). ANT-HSA seemed to retain similar gross polypeptide conformation to that of native HSA. ANT-HSA moved as a single band on slab electrophoresis at the same position as native HSA. This result indicated that ANT-HSA exists as a monomer.

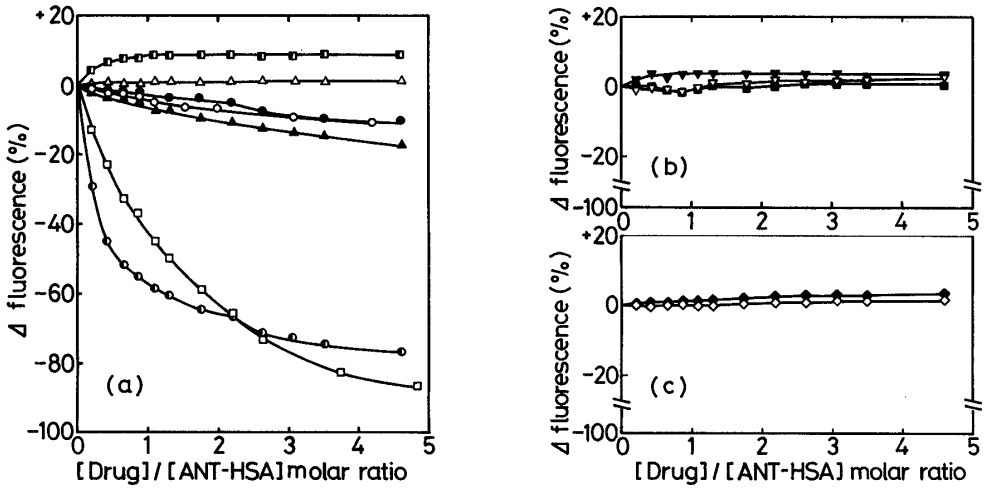

Fig. 1. Fluorescence Quenching of the Anthraniloyl Group of ANT-HSA Induced by Various Drugs at pH 7.0

The degree of quenching was expressed as a percentage of the initial ANT-HSA fluorescence at 425 nm excited at 360 nm. The ANT-HSA concentration was 2.2 μM. The other experimental conditions were as described in Materials and Methods.
(a) ●, DAP; ○, CDO; ◐, FA; △, WA; ▲, APz; □, IM; ◧, PB. (b) ▽, TP; ▼, IP; ■, SDM. (c) ◆, SA; ◇, TB.

Effect of Drug Addition on the Fluorescence Spectrum of ANT-HSA

In order to establish the contribution of Tyr 411 to the drug binding sites of HSA, binding of various drugs (DAP, CDO, APz, FA, IM, SA, IP, TP, TB, SDM, PB and WA) was measured by fluorometry and difference UV spectroscopy. Among the drugs examined here, DAP, CDO, TP, FA and IP were selected as drugs for site II and APz, PB and WA as drugs for site I. SA, TB and SDM may be bound at both sites.

The above drugs were added in increasing amounts to ANT-HSA at pH 7.0 and 20 °C, and quenching of the fluorescence of the anthraniloyl group was measured in 50 mM phosphate buffer (pH 7.0) with an excitation wavelength of 360 nm (Fig. 1). The occurrence of quenching may indicate that drugs interact with ANT-HSA in the proximity of the Tyr 411 residue. The binding of these drugs to ANT-HSA, was confirmed by measuring the UV difference spectra induced by addition of various concentrations of the drugs (Fig. 2). Among

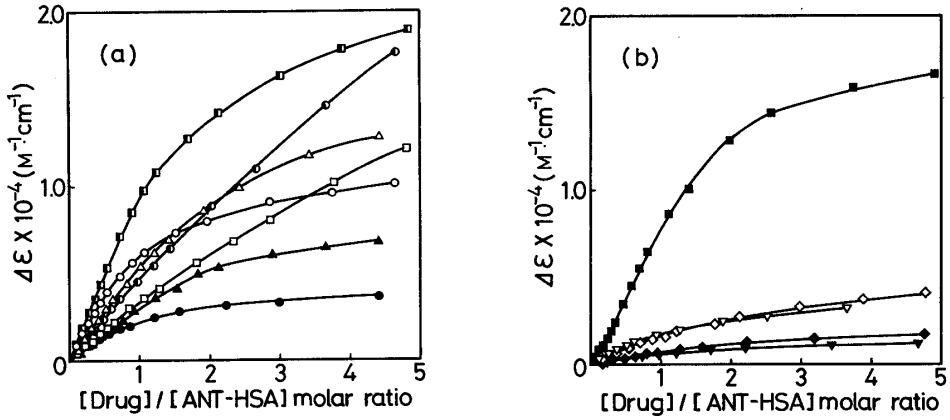

Fig. 2. The Effect of Various Drugs on the Difference UV Absorption of ANT-HSA at pH 7.0

The ANT-HSA concentration was 18.8 μM. The other experimental conditions were as described in Materials and Methods.

(a) ●, DAP ($\Delta\varepsilon_{261}$); ○, CDO ($\Delta\varepsilon_{282}$); ◐, FA ($\Delta\varepsilon_{273}$); △, WA ($\Delta\varepsilon_{332}+\Delta\varepsilon_{299}$); ▲, APz ($\Delta\varepsilon_{358}+\Delta\varepsilon_{309}$); □, IM ($\Delta\varepsilon_{271}$); ◧, PB ($\Delta\varepsilon_{286}$). (b) ▽, TP ($\Delta\varepsilon_{292}$); ▼, IP ($\Delta\varepsilon_{292}$); ■, SDM ($\Delta\varepsilon_{281}$); ◆, SA ($\Delta\varepsilon_{307}$); ◇, TB ($\Delta\varepsilon_{241}$).

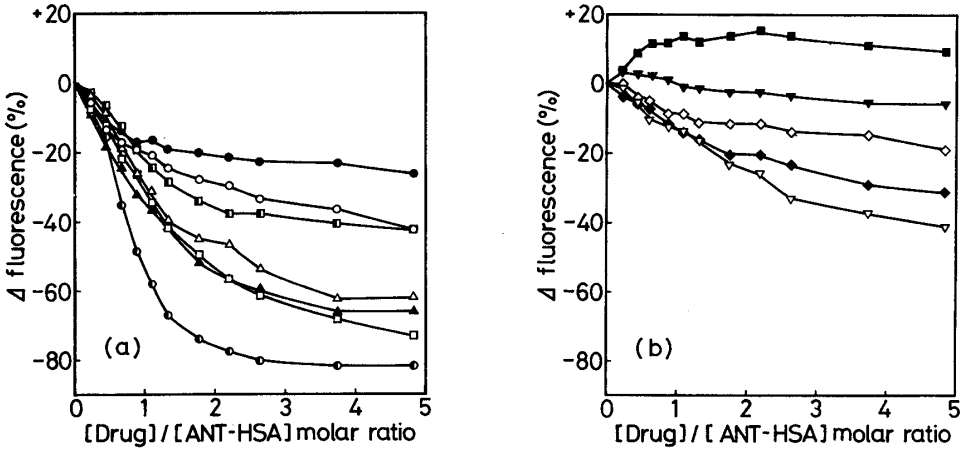

Fig. 3. Quenching of Tryptophan Fluorescence of ANT-HSA Induced by Various Drugs at pH 7.0

The degree of quenching was expressed as a percentage of the initial ANT-HSA fluorescence at 350 nm excited at 295 nm. The ANT-HSA concentration was 2.2 μM. The other experimental conditions were as described in Materials and Methods.

(a) ●, DAP; ○, CDO; ◐, FA; △, WA; ▲, APz; □, IM; ◧, PB. (b) ▽, TP; ▼, IP; ■, SDM; ◆, SA; ◇, TB.

the drugs tested, FA and IM markedly quenched the fluorescence of ANT-HSA at 425 nm even at a drug/ANT-HSA ratio of 1.0. The results indicated that the primary binding sites of FA and IM are near Tyr 411. DAP, CDO and APz quenched the fluorescence of ANT-HSA moderately when added in amounts of more than 1 molar eq.

In contrast, when the interaction of the drugs was examined by difference UV spectroscopy, IP, TP, TB, SDM, SA and WA were found to bind with ANT-HSA at the drug/ANT-HSA ratio of 1.0, even though these drugs did not cause any quenching of ANT-fluorescence emission (Figs. 1 and 2). Therefore it could be concluded that the primary binding sites for these drugs are relatively far from Tyr 411. The binding of PB is somewhat unusual, because the binding caused some enhancement of ANT-fluorescence, though to a very small extent. Since the tryptophan fluorescence of ANT-HSA (excitation at 295 nm, emission at 350 nm) was also quenched by the binding of these drugs except IP and SDM at a molar ratio of 1.0, the primary binding sites of these drugs may also be in close proximity to Trp 214 (Fig. 3).

Discussion

Among the drugs tested, FA and IM markedly quenched the fluorescence of ANT-HSA at 425 nm even at a drug/ANT-HSA ratio of 1.0 (Fig. 1). The results indicated that the primary binding sites of FA and IM are near Tyr 411. However, at a drug/ANT-HSA ratio of 1.0, quenching of the fluorescence of ANT-HSA at 425 nm by drugs such as CDO and DAP was not observed. In the previous paper, we studied the interactions of CDO, WA and PB with NBS-oxidized HSA in which a single tryptophan residue of HSA was oxidized[20] The results indicated that only the binding of CDO with HSA is affected by tryptophan oxidation. Similar NBS-oxidation experiments showed that DAP, TP, TB, SDM and FA bindings were also affected by the oxidation of tryptophan (Sakamoto, Nagata and Irie,[23] unpublished data). Therefore, the primary binding sites of these drugs are near a tryptophan residue and not in close proximity to Tyr 411. Since weak quenching of ANT-HSA-fluorescence was observed at higher drug/ANT-HSA ratios, secondary binding sites of these drugs might involve the Tyr 411 locus.

Ozeki *et al.*[17] and Kurono *et al.*[18,19] classified the drug binding sites of HSA into 3 groups (R, T and U sites) by measuring the inhibition by the drugs of the esterase activity of HSA. Means *et al.*[15] extensively studied the interactions of many drugs with HSA by the same method as described by Ozeki *et al.*[17] and Kurono and Ikeda.[18] They tentatively defined four binding sites on the surface of HSA: (1) bilirubin binding site; (2) palmitate binding site; (3) tryptophan binding site which coincided with or overlaps the binding site for *p*-nitrophenylacetate and includes Tyr 411 (benzodiazepines, FA and IP are classified as drugs which bind to this site); (4) WA, IM, PB and APz binding site. Most drugs which interact with HSA belong to group (3) or (4).

The fluorescence quenching of ANT-HSA described in this paper suggested that FA and IM interact strongly with the anthraniloyl chromophore attached to Tyr 411 or interact with some functional groups located very close to Tyr 411. The results are somewhat contradictory to those of Means *et al.*,[15] who classified IM and FA into Group 4 and Group 3, respectively. The apparent contradiction could be overcome by assuming that many drugs that bind to the tryptophan binding site also interact with anthraniloyl chromophore of the tyrosine 411 residue and not with phenolic chromophore. This assumption requires that the tryptophan binding site and the PB, WA and IM site are located in close proximity.

As can be seen in Fig. 2, the difference UV spectrum induced by addition of FA and IM increased linearly with increasing addition of drugs under the experimental conditions described here. In contrast, the fluorescence quenching induced by IM and FA almost reached

a plateau at the drug/ANT-HSA ratio of two. These results indicated that the second and third binding sites of FA and IM on HSA do not involve the tryptophan residue or Tyr 411 residue.

References

1) C. F. Chignell, *Mol. Pharmacol.*, **5**, 455 (1969).
2) C. F. Chignell, *Mol. Pharmacol.*, **6**, 1 (1970).
3) C. F. Chignell and D. K. Starkwether, *Mol. Pharmacol.*, **7**, 229 (1971).
4) J. J. Vallner, *J. Pharm. Sci.*, **66**, 447 (1977).
5) N. Roosdorp, B. Wänn and I. Sjöholm, *J. Biol. Chem.*, **252**, 3876 (1977).
6) U. Kragh-Hansen, *Pharmacol. Rev.*, **33**, 17 (1981).
7) K. J. Fehske, W. E. Müller and U. Wollert, *Biochem. Pharmacol.*, **30**, 687 (1981).
8) K. J. Fehske, U. Schläfer, U. Wollert and W. E. Müller, *Mol. Pharmacol.*, **21**, 387 (1982).
9) G. Sudlow, D. J. Birkett and D. N. Wade, *Mol. Pharmacol.*, **12**, 1052 (1976).
10) I. Sjöholm, B. Ekman, A. Kober, I. Ljungstedt-Påhlman, B. Seiving and T. Sjödin, *Mol. Pharmacol.*, **16**, 767 (1979).
11) K. J. Fehske, W. E. Müller and U. Wollert, *Biochim. Biophys. Acta*, **577**, 346 (1979).
12) K. J. Fehske, E. Jähnchen, W. E. Müller and A. Stillbauer, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **313**, 159 (1980).
13) G. E. Means and H.-L. Wu, *Arch. Biochem. Biophys.*, **194**, 526 (1979).
14) N. P. Sollenne and G. E. Means, *Mol. Pharmacol.*, **15**, 754 (1979).
15) G. E. Means, N. P. Sollenne and A. Mohamed, *Adv. Chem. Ser.*, **198**, 325 (1982).
16) B. Meloun, L. Moravek and V. Kostka, *FEBS Lett.*, **58**, 134 (1975); P. Q. Behrens, A. M. Spiekerman and J. R. Brown, *Fed. Proc.*, **34**, 591 (1975).
17) Y. Ozeki, Y. Kurono, T. Yotsuyanagi and K. Ikeda, *Chem. Pharm. Bull.*, **28**, 535 (1980).
18) Y. Kurono and K. Ikeda, *Chem. Pharm. Bull.*, **29**, 2993 (1981).
19) Y. Kurono, H. Yamada and K. Ikeda, *Chem. Pharm. Bull.*, **30**, 296 (1982).
20) H. Sakamoto, I. Nagata, K. Kikuchi, M. Aida and M. Irie, *Chem. Pharm. Bull.*, **31**, 971 (1983).
21) N. Hagag, E. R. Birnbaum and D. W. Darnall, *Biochemistry*, **22**, 2420 (1983).
22) Laemmli, *Nature* (London), **227**, 680 (1970).
23) H. Sakamoto, I. Nagata and M. Irie, Abstracts of Papers, 103rd Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1983, p. 433.