Characterization of High Affinity Binding Sites of Non-steroidal Anti-inflammatory Drugs with Respect to Site-Specific Probes on Human Serum Albumin

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A site-oriented study of nine non-steroidal anti-inflammatory drugs (NSAIDs), suprofen, ibuprofen, diclofenac sodium, piroprofen, flurbiprofen, ketoprofen, phenylbutazone, oxyphenbutazone, and ketophenylbutazone on human serum albumin (HSA) was carried out at pH 7.4 by various direct and indirect methods to gain insight into the high affinity binding sites of NSAIDs. The binding was determined by equilibrium dialysis, circular dichroism and fluorescence methods in order to strengthen the results. Irrespective of the method used, close agreement between the binding parameters obtained by the different methods was obtained. A site-oriented description, as revealed by the fluorescent probe displacement method, suggests that site II and site I probes bound to HSA were selectively displaced by NSAIDs with a carboxyl group and without a carboxyl group, respectively. The absence of a carboxyl group of the NSAIDs changed the binding site from site II to site I. Different binding models describing the competitive and independent binding of a NSAID and a site-specific probe bound simultaneously to HSA further describe the respective high affinity binding sites for NSAIDs, thus underscoring the necessity of a carboxyl group in order for a NSAID to bind to site II. The presence of tyrosine, lysine and histidine amino acid residues in the binding of carboxyl group-containing NSAIDs to HSA was evident, whereas tryptophan is believed to take part in the binding of non-carboxyl group-containing NSAIDs. The present findings support the proposal that two separate primary binding sites exist for different NSAIDs; hence, an attempt to correlate the present results with those in literature has been made.

Keywords non-steroidal anti-inflammatory drug; binding site; human serum albumin; probe; independent binding; competitive binding

A wealth of information has been accumulated in the past regarding the binding of drugs to human serum albumin (HSA). Drugs having association constants of $10^5 \text{M}^{-1}$ or larger have a definite influence on the distribution of the drugs.1,2) Though a bewildering array of data has been published concerning the binding of individual ligands and interactions between ligands and those that compete for protein binding,3–5) some order has recently been brought to this mass of information by the recognition that there is a limited number of ligand binding sites on HSA, and each exhibits some degree of specificity or selectivity toward certain ligands. The findings about the rather small number of drug binding sites on of the HSA molecule, and of their sometimes receptor-like properties, emphasize the value of HSA as a convenient model for studying the molecular basis of specific ligand-protein interactions.6) Ligands can not only compete for binding at a specific site, but their binding at one site can also alter (increase or decrease) the strength of interaction between another ligand and the protein at another site.7) When considering ligand binding, it is necessary to recognize that HSA is not a homogeneous protein and does not have a fixed tertiary structure.

The site-oriented approach to drug-albumin binding was first methodically studied by Sudlow et al.,8,9) who described HSA as having two specific anion binding sites, namely site I, also called the warfarin (WF)–azapropazone binding site, and site II, also called the indole–benzodiazepine binding site. The site-oriented approach to drug-albumin binding was further studied by Sjöholm et al.,10) using the polyacrylamide-immobilized HSA binding of 140 drugs. Later this classification was reassessed by Honore et al.,11) by studying the binding of 12 non-steroidal anti-inflammatory drugs (NSAIDs) to HSA by equilibrium dialysis (ED). Their finding regarding the reality of sites I and II does not indicate that these sites are present as preformed sites on the HSA molecule, but that HSA probably forms ad hoc sites, in variable combinations, according to the needs of the individual ligands. Based on data from Sjöholm et al.,10) plus earlier data from Sudlow et al.,8,9) Lin et al.,12) have tried to classify NSAIDs according to their primary binding sites on HSA.

Recently, some works from this laboratory13–15) have shown that NSAIDs of the aryl propionic acid class bind to site II on HSA, but that the methylation of a carboxyl group of the NSAIDs changed the binding site from site II to site I.

In this study, we have used various approaches to study the HSA binding sites of NSAIDs as a class of drugs. Ketoprofen (KP) and ketophenylbutazone (KPB) were chosen as model drugs for this purpose. First, the so-called fluorescent probe displacement, as described by Sudlow et al.,8) was applied to the study of the binding sites. Later, we attempted to locate the site by using the binding model of Kragh–Hansen16) in cases involving two drugs binding to HSA together. Finally, the binding of NSAIDs to different chemically modified HSA was carried out to detect the presence of probable amino acids in the binding site of respective NSAID. The purpose of this study is to attain more exact knowledge about the location, size and composition of HSA binding sites for NSAIDs, which in turn might be helpful for studying the binding sites of NSAIDs with HSA in greater detail.
Materials and Methods

Materials  HSA was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). HSA was defatted with activated charcoal in solution at 0 °C, acidified with H₂SO₄ to pH 3, and then freeze-dried until used. The molecular mass of HSA was assumed to be 66500 Da. HSA gave only one band in sodium dodecyl sulfate-polycrylamide gel electrophoresis (SDS-PAGE). Dansylsarcosine (DNSS) was purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.), Ibuprofen, flurbiprofen (Kaken Pharmaceuticals Co., Tokyo, Japan), suprofen and diclofenac sodium (Tajyo Pharmaceutical Industry Co., Takayama, Japan), KP (Kissei Pharmaceutical Co., Matsumoto, Japan), phenylbutazone, piromphen and oxyphenbutazone (Ciba Geigy Co., Summit, NJ, U.S.A.), WF (Eisai Co., Tokyo, Japan) and KPB (Sanwa Kagaku Co., Mie, Japan) were obtained as pure substances from the manufacturer.

Methods  All experiments were performed in 0.067 M sodium phosphate buffer, pH 7.4.

Fluorescence: Fluorescence measurements were made at 25 °C on a Jasco FP-700 fluorometer (Tokyo, Japan). The intrinsic fluorescence of HSA was obtained at 290 nm. Within the limits of sensitivity, an excitation wavelength was chosen to ensure the least possible absorption of incident light. Emission wavelengths were chosen to give the maximum fluorescence of the probe bound to HSA, with insignificant fluorescence in the buffer. Probes which were chosen had either single binding site or two sites with widely separated dissociation constants. The drug to HSA ratio was kept at 1:1 in order to keep the non-specific binding of probes to a minimum. The percentage of displacement of the probe was determined according to the method of Sudlow et al.¹⁴

\[
F_1 - F_2 \times 100 \quad (1)
\]

Where, \(F_1\) and \(F_2\) represent the fluorescence of the probe plus HSA without the drug and with the drug, respectively.

Binding of Drugs  a) Fluorescence: Fluorescence titrations were used to quench the intrinsic fluorescence of HSA (2 μM) by excitation at 290 nm upon the successive additions of a drug solution (to give a final concentration of 0-10-4 M). The bound fractions of the drug were determined as described by Weber and Young.¹⁷

b) Equilibrium Dialysis (ED); ED experiments were performed using 2 ml Sanko plastic dialysis cells (Fukuoka, Japan). Aliquots (1.5 ml) of various ratios of the drug-HSA mixture (HSA, 40 μM; drug, 20-400 μM) were dialyzed at 25 °C for 13 h against the same volume of buffer solution. It was demonstrated at the concentration used that there was no significant adsorption of the drugs to the dialysis membrane. Further control experiments with protein-free solutions showed that visking membranes were fully permeable to all drugs and that equilibrium was established within the designated period of time. After equilibrium was reached, the free concentration was determined by HPLC. The HPLC system consisted of a Hitachi 655-A1 pump and either a Hitachi 655A variable UV monitor or a Hitachi F-1000 variable fluorescence monitor. A column of LiChrosorb RP-18 (Cica Merck, Tokyo, Japan) was used as a stationary phase. The detector was set at 257 nm with a sensitivity of 0.005 A.U.F.s for KP and DNSS, and at 253 nm with the same sensitivity of 0.005 A.U.F.s for KPB. The excitation and emission wavelengths for the assay of both were set at 310 and 400 nm, respectively. The mobile phases consisted of an acetate buffer (pH 4.5)–acetonitrile (63:37, v/v) for KP, DNSS and KPB, and a 30 mm phosphate buffer (pH 7.4)–acetonitrile (80:20, v/v) for WF.

c) Circular Dichroism (CD); CD experiments were performed on a Jasco J-600 spectropolarimeter (Tokyo, Japan) using 10 mm cells at 25 °C. HSA (40 μM) was titrated by the successive addition of the drug (2-400 μM). The induced CD ellipticity is defined as the CD of the drug-HSA mixture minus the CD of HSA alone at the same wavelength, and it is expressed in degrees. Free and bound concentrations of the drugs were calculated according to the method of Rosen.¹⁸

Preparation of HSA Derivatives  Tryptophan (Trp), tyrosine (Tyr), lysine (Lys) and histidine (His) amino acid residues were modified by, 2-hydroxy-5-nitrobenzyl bromide (HNBBr), tetramethylthromine (TNTM), succinic acid (SA) and methylene blue (MB), respectively, according to the methods described before.¹³⁻¹⁵

Data Analysis  All the binding parameters were estimated by fitting the experimental data to the following Scatchard equation¹⁰ using a non-linear squares computer program (MULTI program),²⁰

\[
r/D_i = nK - rK \quad (2)
\]

where \(r\) is the number of moles of bound drug per mole of protein, \(n\) is the number of binding sites per protein molecule, \(K\) is the association constant, and \(D_i\) is the free drug concentration.

The binding of two ligands to the protein is sometimes very complicated and cannot be explained by a simple competition interaction alone. In the present study, on the basis of a 1:1 complex formation by all compounds bound to HSA, we treated data according to the assumption that the interaction of two ligands simultaneously bound to HSA had taken place according to the interaction models illustrated in Chart 1.¹⁰

Model 1 exemplifies a competitive binding of two ligands, A and B, in general, to a single site on a protein molecule using the following equations,

\[
r_{A} = \frac{[PA]}{[P]} = \frac{K_d[A]}{1 + K_d[A] + K_d[B]} \quad (3)
\]

\[
r_{B} = \frac{[PB]}{[P]} = \frac{K_d[B]}{1 + K_d[B] + K_d[A]} \quad (4)
\]

where \([P]\) is the total HSA concentration, \([A]\) and \([B]\) are the concentrations of the ligands; \([PA]/[P]\) and \([PB]/[P]\) are the average numbers of mol of A and B bound per mol of protein, respectively; and \(K_d\) and \(K_d\) are the association constants of ligands A and B, respectively. In the present context, A and B stand for KP and KPB, respectively.

Model 2, which was adopted by Krahl-Hansen,¹⁰ represents the case of two drugs binding to HSA together. This model is applicable to those cases where two ligands bind to two different high affinity binding sites on a protein. This model represents a protein possessing one binding site for ligand A and one binding site for ligand B. The different \(K\) values stand for respective association constants. If A and B bind independently to P, then \(K_d\) is the same as \(K_d\), and \(K_d\) is equal to \(K_d\). Apart from the independent binding, in other situations,

\[
k_d = xK_d \quad \text{and} \quad k_{AB} = xK_d
\]

In this case, the association constants are affected to the same extent, where \(x\) is defined as a coupling constant, which is the factor by which the binding of one ligand to its high affinity binding site affects the high affinity binding constant for the binding of the other ligand. To ensure an accurate analysis of the competitive binding studies, the following precautions were taken: (1) the number of mol of ligand bound per mol of protein was 0.5 or less to minimize ligand binding to secondary sites, and (2) the binding of both ligands was determined simultaneously.

If A and B bind independently to HSA, then \(x = 1\). Other cases, where \(x = 0\), \(x \gg 1\) and \(x < 1\), indicate competitive, cooperative and anticoporative binding, respectively. The value of \(x\) was calculated as follows¹⁰:

The sum of the total protein concentration is equal to the sum of the concentrations of \([P]\), \([PA]\), \([PB]\) and \([BPA]\).

\[
[P] = [P] + [PA] + [PB] + [BPA] \quad (5)
\]

The relationship can be transformed to:

\[
\]

The concentration of bound A \([A]\) is given by:

\[
\]

where \([A]\) and \([A]\) are total and free concentrations of ligand A. Now, substituting Eq. 7 from Eq. 6 gives:

\[
[P] - [A] = [P] + K_d [B] + [P] \quad (8)
\]
Since $[P]$, $[A_1]$, $[A_2]$, and $K_h$ are known, the value of $[F]$ can easily be calculated. Now, putting the calculated value of $[F]$ and other known values for $K_h$ and $[A_2]$ into Eq. 6, the value of $x$ can be obtained.

**Results**

The chemical structures of NSAIDs used in this study are shown in Fig. 1.

**Displacement of Fluorescent Probes from HSA by Drugs**
The displacement of fluorescent probes from their binding sites on HSA of nine NSAIDs was studied by the method described by Sudlow. As representative examples, the probe displacements by KP and KPB are shown in Fig. 2. As can be seen in Fig. 2, the fluorescence of a site I probe bound to HSA was remarkably decreased by KPB; on the other hand, the fluorescence of a site II probe bound to HSA was more decreased by KP. Table I shows the extent of displacement of site specific probes by various NSAIDs.

**Binding of KP and KPB to HSA**
As representative examples, the binding of two drugs, KP and KPB, was studied individually using both direct and indirect methods. KP and KPB were chosen as representative compounds because the former compound contains a carboxyl group, whereas, the latter has no carboxyl group. The number of binding sites ($n$) and corresponding association constants ($K$) were calculated by the Scatchard equation. Results obtained are summarized in Table II.

The binding of KPB to HSA induced a monophasic Cotton effect by exhibiting a positive maximum at 285 nm. When a fixed concentration of HSA was titrated with increments of KPB, the extrinsic ellipticity of this peak increased. The induced ellipticities at 285 nm were plotted against corresponding ratios of KPB to HSA. Accordingly, the free and bound fractions were calculated. Though the

<table>
<thead>
<tr>
<th>Drug</th>
<th>Displacement of bound probe (%)</th>
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<tbody>
<tr>
<td></td>
<td>WF</td>
</tr>
<tr>
<td>KP</td>
<td>9.50</td>
</tr>
<tr>
<td>Suprofen</td>
<td>11.1</td>
</tr>
<tr>
<td>Flurbiprofen</td>
<td>0.00</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>0.00</td>
</tr>
<tr>
<td>Piroprofen</td>
<td>14.0</td>
</tr>
<tr>
<td>Diclofenac sodium</td>
<td>27.1</td>
</tr>
<tr>
<td>KPB</td>
<td>30.9</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>54.7</td>
</tr>
<tr>
<td>Oxyphenbutazone</td>
<td>42.2</td>
</tr>
</tbody>
</table>

Following concentrations were used. HSA, $2 \times 10^{-6}$ M, probe, $2 \times 10^{-4}$ M, and drug, $10 \times 10^{-4}$ M.

![Chemical Structures of the Compounds Used in This Study](image)

![Effect of KP (A) and KPB (B) on the Fluorescence of Site Marker Probes](image)

HSA and probe were $2 \mu$ in 0.067 M phosphate buffer (pH 7.4). The mean data of three experiments are shown. Site marker probes used were: WF (●, site I marker), DNSS (▲, site II marker).
TABLE II. Binding Parameters of KP and KPB to HSA as Estimated by Different Methods at pH 7.4 and 25°C

<table>
<thead>
<tr>
<th>Methods</th>
<th>KP</th>
<th>KPB</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$n_1$</td>
<td>$K_1$ ($\times 10^8$ M$^{-1}$)</td>
</tr>
<tr>
<td>ED</td>
<td>1.0</td>
<td>2.5</td>
</tr>
<tr>
<td>CD</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Fluorescence quenching</td>
<td>1.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Fig. 3. Binding of KP in the Presence of KPB (A) and Vice Versa (B) to HSA by ED at pH 7.4 and 25°C.

(A) Binding of KP (4–20 μM) to HSA (40 μM) (○) in the presence of KPB (20 μM). (B) Binding of KPB (4–40 μM) to HSA (40 μM) (●) in the presence of KP (20 μM). The broken line curves (---) represent the theoretical curves for binding of the two ligands assuming competition for a common binding site on the protein. The solid line curves (——) represent the theoretical curves for independent binding of the two ligands.

Fig. 4. Binding of KP in the Presence of DNNS (A) and Vice Versa (B) to HSA by ED at pH 7.4 and 25°C.

(A) Binding of KP (4–20 μM) to HSA (40 μM) (○) in the presence of DNNS (20 μM). (B) Binding of DNNS (4–20 μM) to HSA (40 μM) (●) in the presence of KP (20 μM). The broken line curves (---) illustrate the theoretical curve for competitive binding between the two ligands, whereas the solid line curves (——) represent the theoretical curves for independent binding of the two ligands.

Fig. 5. Binding of KPB in the Presence of WF (A) and Vice Versa (B) to HSA by ED at pH 7.4 and 25°C.

(A) Binding of KPB (4–40 μM) to HSA (40 μM) (●) in the presence of WF (20 μM). (B) Binding of WF (4–32 μM) to HSA (40 μM) (○) in the presence of KPB (20 μM). The broken line curves (---) represent the theoretically calculated curves assuming competition between the ligands for a common binding site on the protein. The solid line curves (——) represent the theoretical curves for independent binding of the ligands.

TABLE III. Percentage of Reacted Amino Acid Residues in the Modified HSA

<table>
<thead>
<tr>
<th>Modified HSA</th>
<th>Reacted amino acid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNBB-treated</td>
<td>93 0.0 2.3 1.5</td>
</tr>
<tr>
<td>TNM-treated</td>
<td>0.0 10 0.0</td>
</tr>
<tr>
<td>SA-treated</td>
<td>0.0 3.2 64 0.0</td>
</tr>
<tr>
<td>MB-treated</td>
<td>60 0.8 2.0 10</td>
</tr>
</tbody>
</table>

The number in parentheses represents the number of amino acids per HSA molecule.

estimation of the binding of KPB to HSA by the CD method was feasible, we unfortunately could not measure the binding of KP to HSA by the CD method due to the lack of a noticeable extrinsic Cotton effect of the KP-HSA complex.

The binding of both KP and KPB to HSA quenched the intrinsic fluorescence of HSA. The fluorescence intensities were plotted against a corresponding ligand concentration at a fixed HSA concentration to calculate the bound molar ratios of the ligand to HSA.

From the dialysis data, two successive saturable processes were observed when both KP and KPB were bound to HSA. The best fitting values for the binding parameters are summarized in Table II.

Binding of KP in the Presence of KPB and Vice Versa

In Fig. 3A, it is seen that the binding of KP in the presence of KPB is far from the theoretical curve, assuming competition, but too close to the theoretical curve, assuming the independent binding of the two drugs. Figure 3B shows the results of the reverse experiments. The binding of KPB in the presence of KP was very close to the theoretically calculated curve assuming the independent binding of the two drugs, but was far from the theoretical curve, assuming competition between the ligands for a common binding site.

Binding of KP to HSA in the Presence of Site II Specific Probe and Vice Versa

Figure 4A represents the binding of KP to HSA in the presence of a site II specific probe, DNNS. As shown in Fig. 4A, the experimental data of the binding of KP to HSA in the presence of DNNS did fit well with the theoretical curve assuming competition between the drug and the probe. The reverse experiment (Fig. 4B) also suggests that the binding of DNNS to HSA
in the presence of KP was competitive.

**Binding of KP to HSA in the Presence of Site I Specific Probe and Vice Versa** Figure 5A represents the binding of KP to HSA in the presence of WF and *vice versa* (5B). In both cases, the observed data fit well the theoretical curve assuming competitive binding between KP and WF as predicted from the results in Table I and Fig. 2. In the present study, the value of $x$ was always either 0 or 1, indicating competitive and independent binding, respectively, between the two ligands bound simultaneously to HSA.

**Binding of KP and KPB to HSA Derivatives** The results in terms of the percentage of reacted amino acid residues, with native HSA taken as 0%, are shown in Table III. To further investigate which amino acid(s) are involved in the specific binding sites of KP and KPB on HSA, the binding of KP and KPB to various modified HSA was carried out using the ED method. The results are shown in Fig. 6. However, we did not get a very clear idea from this experiment, but the contribution of Tyr, Lys, and His amino acid residues was apparent in the binding of KP to HSA, whereas Trp and Lys were found to play an important role in the binding of KPB to HSA.

**Discussion** The three-dimensional structure of crystalline HSA is now known as the result of X-ray crystallographic study at a resolution of 2.8 Å.\(^1\)\(^2\) Despite the knowledge of this crystal structure map, it is nowadays considered to be a major step forward in the detailed understanding of HSA, X-ray crystallographic studies, as yet, have failed to provide detailed information regarding the molecular basis for the unique ligand binding capabilities of HSA. Consequently, in addition, other methods are usually employed in order to locate and characterize drug binding sites in HSA.

**Characterization of a High Affinity Binding Site of NSAIDs by Fluorescent Probe Displacement Method** In the present study, the high affinity binding sites of nine NSAIDs with different chemical structures to defatted HSA was studied using the method of Sudlow.\(^8\) From the preliminary study, using the probe displacement method, it was revealed that site I and site II probes were selectively displaced by NSAIDs. Suprofen, ibuprofen, flurbiprofen, diclofenac sodium, and KP were predominantly bound to site II or the benzodiazepine site on HSA, whereas, oxyphenbutazone, phenylbutazone and KPB were primarily bound to site I or the warfarin site on HSA. Further, this conclusion also agrees with the structural features defined by Sudlow *et al.*\(^9\) NSAIDs, possessing a carboxyl group at the end of the molecule away from the non-polar center, such as in KP, displaced site II probes more prominently than site I probes, whereas, site I probes were displaced significantly by NSAIDs which have no carboxyl group. This suggests that the high affinity binding site of NSAIDs seems to be different between carboxylic and non-carboxylic compounds. In fact, the importance of the carboxylic group of NSAIDs for binding to site II has been emphasized by some reports published from this laboratory.\(^13\)\(^\text{15}\)

**Characterization of a High Affinity Binding Site of a NSAID with Respect to Another NSAID or Site-Specific Probes Using a Generalized Form of Binding Model** The probe displacement method described by Sudlow *et al.* is an indirect method and does not necessarily explain the type of binding in the presence of another ligand. Again, many authors\(^2\)\(^\text{12}\)\(^\text{12}\)\(^\text{24}\) have criticized the "site binding" model as proposed by Scatchard,\(^19\) which addresses only independent binding but does not include cooperative binding. A stepwise equilibrium (or stoichiometric) model has been proposed\(^1\)\(^1\)\(^1\)\(^1\)\(^1\)\(^1\)\(^1\)\(^1\)\(^1\)\(^1\)\(^1\)\(^1\)\(^1\) instead, which defines equilibrium in terms of individual species in solution, possessing $n$ mol of bound ligand, regardless of specific physical site, or addresses the site to which they are bound on each molecule and can easily accommodate cooperative interactions. However, the equations for the model require complex computer solutions, and the equations for this model tend to be ill conditioned, either in the absence or in the presence of competing ligands. On the other hand, the site binding model as suggested by Krágh–Hansen\(^16\) does not count these limitations. Therefore, in the present study, we analyzed our binding data using Krágh–Hansen's model which completely accounted for all factors and fit the data very well. Further, we tried to show whether the results obtained by this model support the data obtained previously by the method of Sudlow *et al.*\(^9\) As we can see from Table II, there was good agreement between the results obtained by the different methods, suggesting the accuracy and validity of the binding parameters as measured by the different methods. Later on, the binding of KP in the presence of KPB and *vice versa* to HSA was studied using

Fig. 6. Binding of KP (A) and KPB (B) to Native HSA and Various Modified HSA by ED at pH 7.4 and 25°C

(A) Native HSA; (B) Trp-modified HSA; (C) Lys-modified HSA; (D) Tyr-modified HSA; and (E) His-modified HSA. Following concentrations were used: native HSA, modified HSA, 10 μM; KP and KPB, 10 μM.

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the binding model of Kragh-Hansen. As can be seen in Fig. 3, the binding of KP in the presence of KBP and vice versa to HSA was independent, suggesting the validity of the previous finding by site-specific probe displacement method, that KP and KBP bind to two distinct binding sites, site II and site I, respectively, on HSA. Further, the relation of a high affinity binding site of KP with respect to a site II specific probe, DNSS, was investigated according to the binding scheme shown in model 2. Competitive binding studies of KP with DNSS (Fig. 4A) and DNSS with KP (Fig. 4B) revealed that the two ligands compete for a common high affinity binding site on HSA. Binding of DNSS or KP to HSA diminishes the simultaneous high affinity binding of either KP or DNSS. The decrease in binding was found to be reciprocal. The decrease in binding might be due to a factor which is referred to as a coupling constant, which affects the association constants of two different ligands during simultaneous binding to HSA. By contrast, the binding of KP in the presence of WF and vice versa were best described by assuming, in accordance with the model, that KP and WF interact with two different high affinity binding sites on HSA (data not shown).

When the binding of KBP with respect to the site I specific probe, WF, was brought into consideration, it was found that there was direct competition between KBP and WF for a common binding site on HSA (Fig. 5A). The converse experiments also showed that KBP and WF bind to a common binding site on HSA (Fig. 5B). However, when a site II specific probe was considered with respect to the binding of KP and vice versa to HSA (data not shown), the binding model clearly demonstrated that the bindings were independent of each other, implying the fact that the two ligands bind to two different sites on HSA.

**Characterization of Binding Site by Amino Acid Residue Modification of HSA** It is well demonstrated that some amino acid residues are specifically involved in drug binding sites. The major and minor parts of the site II binding site are believed to be located in a cyanoogen bromide fragment C (residues 124–298), and in fragment A (residues 299–585), respectively. The presence of His 146, Lys 199 in fragment C and Tyr 411 in fragment A 28–30 was confirmed by several laboratories. In contrast to site II, much less is known about the location of site I. However, Trp 214 is considered to be part of the site I area, 31 and at least one part of this binding site is believed to be located in the cyanoogen bromide fragment C. 32

Some further information about the location of high affinity binding sites for NSAIDs is available from the amino acid residue modification studies, as was performed in this study. Our present data indicate that Tyr, Lys and His take up an important part in the binding of those NSAIDs which bind to site II, whereas, in addition to Trp, Lys also plays an important role in the binding of NSAIDs to site I. These data do not contradict the results obtained by either fluorescent probe displacement or the binding model as described by Kragh–Hansen. According to Sjödin et al., 33 a part of site II is located in a large, trypsin-resistant fragment of HSA (residues 192–582). This fragment though contains Tyr 411 and Lys 199, but not His 146, which partially contradicts our results. But at the same time, it should be remembered that it is difficult to decide whether the failure to find binding to a specific fragment depends on the location of the binding site in another part of HSA or on a loss or destruction of the binding site due to conformational changes of the fragments investigated. 32 The present results are in close agreement with those reported by He and Carter, 211 who have shown by X-ray crystallographic study of HSA that the so-called site I is located in subdomain IIA, and site II in subdomain IIIA. According to these authors, Trp 214, leucine 203 and phenylalanine 211 are present in subdomain IIA, whereas Tyr 411 and arginine 410 are present in subdomain IIIA. To derive more detailed information regarding this, the modification study of some more important amino acid residues is warranted.

**References**