Interaction Mechanism Between Indoxyl Sulfate, a Typical Uremic Toxin Bound to Site II, and Ligands Bound to Site I of Human Serum Albumin

Toshiaki Sakai,[1] Keishi Yamasaki,[1] Tomoko Sako,[1] Ulrich Kragh-Hansen,[1,2] Ayaka Suenaga,[1] and Masaki Otagiri[1,3]

Received November 29, 2000; accepted December 12, 2000

Purpose. The study was performed for clarifying the mechanism of interaction between indoxyl sulfate (IS), a typical uremic toxin bound to site II, and site I-ligands when bound to human serum albumin (HSA). The effect of the N to B transition on the interactions was also examined.

Methods. Quantitative investigation of the relations between ligands bound to HSA was performed by equilibrium dialysis, and the binding data were analyzed on the basis of a theoretical model for simultaneous binding of two ligands.

Results. The high-affinity binding constants for the site I-ligands warfarin (WF) and dansyl-L-asparagine (DNSA) increased with increasing pH, whereas those for the site II-ligands IS and dansylsarcosine (DNSS) were hardly affected by pH. Mutual displacement experiments showed that even though IS binds to site II it influenced binding of DNSA at the azapropazone binding area in site I. By contrast, it is unlikely that IS affects the WF binding area of site I. Furthermore, pH-profiles showed that the interaction between IS and DNSA was very sensitive to the N to B transition: "competitive-like" strong allosteric regulation was observed for binding of the two ligands to the N conformer (pH 6.5), whereas in the B conformation (pH 8.5) binding of these molecules was nearly "independent".

Conclusions. The present data provide useful information for elucidating a potential mechanism of interaction between drugs and endogenous substances including uremic toxins.

KEY WORDS: human serum albumin; indoxyl sulfate; dansyl-L-asparagine; high-affinity binding site; allosteric interaction; N to B transition.

INTRODUCTION

In patients with chronic renal failure defective drug binding occurs in plasma. This is the case especially for acidic drugs and takes place even in the presence of a normal concentration of albumin, which is the major carrier protein for that type of drugs in the blood (1). The condition may in part be caused by accumulation of endogenous substances, the so-called uremic toxins, which are known to be strongly bound to albumin (2,3). Recently, we have characterized the binding sites for several uremic toxins and found that indoxyl sulfate (IS), indole-3-acetic acid (IA) and hippuric acid (HA) bind with high affinity to site II, whereas 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) is primarily bound to site I of human serum albumin (HSA) (4). In addition, the interaction between IS and IA was found to be fully competitive, whereas no interaction was observed between albumin-bound IS and CMPF. These findings are in accordance with the recent X-ray crystallographic data of HSA which shows that the two major ligand binding sites, sites I and II, are located within specialized cavities in two separate subdomains, namely subdomain IIA and IIIA, respectively (5,6). However, very recently we have found a competitive-like antagonism between binding of dansyl-L-asparagine (DNSA), which is a site I-probe, and ibuprofen and diazepam which bind with a high affinity to site II (7). Furthermore, we have observed that the mutual interactions were effected by the N to B transition, a conformational change of HSA taking place in the pH-interval 6–9. These findings led us to the idea that perhaps binding of site I-drugs could be affected not only by CMPF but also by, for example, the site II-ligand IS. The N to B transition could also have an influence on a potential effect of IS. In the present work, we have examined these questions hoping that the information gained can make more clear the mechanism of drug-protein interactions in renal disease.

MATERIALS AND METHODS

Materials

HSA was a gift from the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). Using the method described by Chen (8), it was defatted with activated charcoal in solution at 0 °C, acidified with H_2SO_4 to pH 3, deionized and then freeze-dried. The protein gave only one band on SDS-PAGE, and the molecular mass was assumed to be 66500 Da. DNSA and dansylsarcosine (DNSS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Warfarin (WF) was supplied by Eisai Co. (Tokyo, Japan). IS was purchased from Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals were of analytical grade, and all solutions were prepared in deionized and distilled water. Phosphate buffer, 67 mM, was used as a standard buffer, and it was made from sodium phosphate dibasic and sodium phosphate monobasic salts.

Equilibrium Dialysis

Equilibrium dialysis experiments were performed using 2 mL Sanko plastic dialysis cells (Fukuoka, Japan). The two cell compartments were separated with a Visking cellulose membrane. Aliquots with various molar ratios of ligand and HSA (40 μM) were dialyzed at 25°C for 15 h against the same volume of buffer solution. After equilibrium was reached, the concentrations of free ligands in the buffer compartment were determined by a HPLC system consisting of a Hitachi L-6000 intelligent pump and a Hitachi F 1050 variable fluorescence monitor. A LiChrosorb RP-18 (Cica Merk, Tokyo, Japan) column was used as the stationary phase. The mobile phase

[1] Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oehonmachi, Kumamoto 862-0973, Japan.
[2] Guest professor at Kumamoto University. Permanent address: Department of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark.
[3] To whom correspondence should be addressed. (e-mail: otagirim@gpo.kumamoto-u.ac.jp)

ABBREVIATIONS: HSA, human serum albumin; IS, indoxyl sulfate; IA, indole-3-acetic acid; HA, hippuric acid; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid; WF, warfarin; DNSA, dansyl-L-asparagine; DNSS, dansylsarcosine.

0724-8741/01/0400-0520$19.50/0

consisted of 0.2 M acetate buffer (pH 4.5)-acetonitrile (40 : 60 v/v for WF, DNSA and DNSS; 65 : 35 v/v for IS). For the assay, the excitation and emission wavelengths were 300/400 nm, 330/550 nm, 310/550 nm and 280/375 nm for WF, DNSA, DNSS and IS, respectively. Adsorption of ligands onto the dialysis membrane and/or the dialysis apparatus was negligible since no adsorption was detected in equilibrium dialysis experiments without albumin.

Binding Data Analysis

The values of the binding parameters were estimated by fitting the experimental data to the following equation using a non-linear least squares computer program (MULTI program) (9):

$$r = \frac{[D_b]}{[P_t]} = \sum_{i=1}^{j} \frac{n_i K_i [D_f]}{1 + K_i [D_f]} \quad (1)$$

where r is the number of molecules of ligand bound per molecule of protein. $[D_b]$ and $[D_f]$ are the bound and unbound ligand concentrations, respectively, and $[P_t]$ is the protein concentration. $K_t$and $n_i$are the association constant and the number of binding sites in the ith class of binding sites, respectively.

Data for simultaneous binding of one molecule of each of two ligands, A and B, to one protein, P, were analyzed by the thermodynamic cycle model with four equilibria (10). In this model, the concentration of bound ligand A is the sum of $[PA]$ and $[BPA]$, which are the concentration of protein binding only ligand A and protein binding both ligands, respectively. The sum, $[A_b]$, can be calculated as follows:

$$[A_b] = K_A [P_f] [A_f] + K_{BA} K_B [P_f] [A_f] [B_f] \quad (2)$$

where K_A and K_B are the binding constant of ligand A and B, respectively, while K_{BA} is the binding constant of ligand A in the presence of ligand B. $[A_f]$ and $[B_f]$ are the free concentration of ligand A and B, respectively, and $[P_f]$ is the concentration of free protein.

Furthermore, the concentration of total protein, $[P_t]$,is the sum of $[P_f]$, $[PA]$, $[BPA]$ and $[PB]$, the latter denoting the concentration of protein binding only ligand B. $[P_t]$ is given by:

$$[P_t] = [P_f] + K_A[P_f][A_f] + K_B[P_f][B_f] + K_{BA}K_B[P_f][A_f][B_f] \quad (3)$$

Now, the fractional degree of saturation, r_A, of the binding site for ligand A can be obtained by dividing Eq. 2 with Eq. 3

$$r_A = \frac{[A_b]}{[P_t]} = \frac{K_A[A_f] + K_{BA}K_B[A_f][B_f]}{1 + K_A[A_f] + K_B[B_f] + K_{BA}K_B[A_f][B_f]} \quad (4)$$

In a similar way, fractional degree of saturation, r_B, of the binding site for ligand B can be described by the following equation:

$$r_B = \frac{[B_b]}{[P_t]} = \frac{K_B[B_f] + K_{AB}K_A[A_f][B_f]}{1 + K_A[A_f] + K_B[B_f] + K_{AB}K_A[A_f][B_f]} \quad (5)$$

In this equation, $[B_b]$ and K_{AB} are the concentration of bound ligand B and the binding constant of ligand B in the presence of ligand A, respectively.

If the thermodynamic cycle is fast, then the following equation is obeyed (10):

$$K_{BA}/K_A = K_{AB}/K_B = \chi \quad (6)$$

where χ is the coupling constant. Independent binding of the two ligands is characterized by $\chi = 1$, while a competitive interaction results in $\chi = >0$. $\chi > 1$ and $0 < \chi < 1$ express cooperative and anti-cooperative interaction between ligands A and B on the protein, respectively. Eqs. 4 and 5 can be modified by using Eq. 6 as follows:

$$r_A = \frac{K_A[A_f] + \chi K_A K_B[A_f][B_f]}{1 + K_A[A_f] + K_B[B_f] + \chi K_A K_B[A_f][B_f]} \quad (7)$$

$$r_B = \frac{K_B[B_f] + \chi K_B K_A[A_f][B_f]}{1 + K_A[A_f] + K_B[B_f] + \chi K_B K_A[A_f][B_f]} \quad (8)$$

RESULTS

Binding of Ligands to HSA at Various pH-Values

Table I shows the effect of pH on binding of IS to HSA as analyzed by using Eq.1 and assuming two independent binding classes. The results given in this and the following table are average values (±S.D.) for at least four experiments. As seen in Table I, there was only a small effect of pH on the two binding classes, a finding indicating that binding of IS to HSA is almost pH-independent. The influence of pH on the primary binding constants of WF, DNSA and DNSS was also investigated (Table II). The high–affinity binding constants for WF and DNSA, site I ligands, increased with increasing pH, whereas that of DNSS, a site II probe, was hardly affected. The present findings (Tables I and II) propose that the structure of subdomain IIA is much more influenced by the N to B transition than that of subdomain IIIA.

Interaction Mechanism Between IS and Site Markers at pH 7.4

Binding of IS in the presence of WF, DNSS or DNSA was studied at relatively low concentrations, and the data were analysed by using the primary binding constants of Tables 1 and 2 and the model for simultaneous binding of two ligands to a protein described in Materials and Methods (Fig.1). It was found that binding of IS in the presence of WF (Fig.1b) as well as binding of WF in the presence of IS (Fig.1d) apparently follow the curves for independent ligand binding. Binding of IS in the presence of DNSS (Fig.1b) and vice versa (Fig.1d) seem to fit well to the theoretical curves assuming competitive binding of the two ligands. The interaction between IS and DNSA was clearly different from the IS-WF interaction even though both DNSA and WF are site I probes. The experimental data for the IS-DNSA system (Figs.1a and c) were clearly distinct from the theoretical curves assuming independent binding and competitive binding between the two ligands. Consequently, the binding data

Table I. Effect of pH on Binding of Indoxyl Sulfate to HSA at 25 °C

pH	n_1	K_1 (10^5 M^{-1})	n_2	K_2 (10^4 M^{-1})
6.5	1.0	10.1 ± 3.2	2.0	1.1 ± 0.6
7.4	1.0	9.1 ± 2.9	3.0	0.8 ± 0.4
8.5	1.0	8.1 ± 3.1	3.0	0.8 ± 0.5

Table II. Effect of pH on Binding of WF, DNSA, and DNSS to HSA at 25 °C

	pH 6.5		pH 7.4		pH 8.5	
Probe	n	K (10^5 M^{-1})	n	K (10^5 M^{-1})	n	K (10^5 M^{-1})
WF	1.0	1.6 ± 0.7	1.0	2.3 ± 0.6	1.0	5.0 ± 1.0
DNSA	1.0	2.8 ± 0.3	1.0	3.0 ± 0.9	1.0	6.0 ± 1.5
DNSS	1.0	14.8 ± 3.0	1.0	14.5 ± 3.1	1.0	13.6 ± 1.5

were described assuming mutual anti-cooperativity between albumin-bound IS and DNSA with a χ value of 0.53.

Effect of pH on the Interaction Between IS and DNSA

Since mutual interactions exist between IS and DNSA even though they bind with high affinity to two different sites of albumin simultaneous binding of the ligands was studied in greater detail. Thus, Figs. 2 and 3 show the results of a quantitative analysis of mutual displacement between IS and DNSA at pH 6.5 and 8.5, respectively. The constants for binding of these ligands to their primary sites at pH 6.5 and 8.5 are given in Table I and II, and they were used for this analysis. As shown in Fig. 2, the binding isotherm of IS in the presence of DNSA at pH 6.5, and vice versa, was qualitatively close to the theoretical curve assuming competition between these two ligands for a common site. In contrast (Fig. 3), at pH 8.5 the experimental data almost fit the simulated curve made on the assumption that IS and DNSA bind independently to HSA. Fig. 4 shows the influence of pH on the values of X calculated for the mutual interactions between IS and DNSA. It is seen that the χ values increased almost linearly from a value close to the lower limit level to a value which is near the higher limit level for binding inhibition. The experimental results of the interactions between IS-WF and IS-DNSS at pH 6.5 and 8.5 showed behaviors similar to those at pH 7.4 (data not shown).

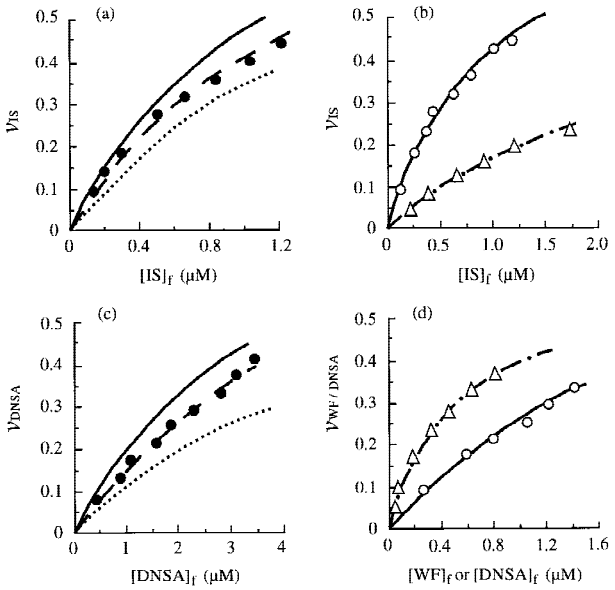

Fig. 1. Binding of IS, DNSA, WF and DNSS to HSA in the presence of another ligand at pH 7.4 and 25 °C. (a), Binding of IS (4–24 μM) to HSA (40 μM) in the presence of DNSA (24 μM) (•). (b), Binding of IS (4–18 μM) to HSA (40 μM) in the presence of WF (24 μM) (○) or DNSS (20 μM) (△). (c), Binding of DNSA (4–16 μM) to HSA (40 μM) in the presence of IS (20 μM) (•). (d), Binding of WF (8–16 μM) (○) or DNSS (4–14 μM) (△) to HSA (40 μM) in the presence of IS (20 μM). The results are means of at least four experiments. (——), Theoretical curves assuming independent binding of the two ligands. (- - - -), Theoretical curves for IS (a) and DNSA (c) binding assuming anti-cooperative interaction between the two ligands (χ = 0.53).(...), Theoretical curves for IS (a) and DNSA (c) binding assuming competition between the two ligands for a common binding site. (– – – –), Theoretical curves for IS (b) and DNSS (d) binding assuming competition between the two ligands for a common binding site.

DISCUSSION

Mutual interactions between two different ligands simultaneously binding to albumin can show as independent or competitive binding, or the binding can be influenced by heterotropic allosteric interactions. Since the vast majority of substances bind with high affinity to either site I or II, many examples of competitive binding of endogenous and/or exogenous substances to either one of these sites have been found (5,6,11,12). Independent binding, on the other hand, usually exists when ligands bind separately to site I and II, and the first documented case was binding of bilirubin (site I) and diazepam (site II) (13). The many cases of independent binding has led to the widely accepted point of view that no or only weak interactions exist between site I and II of HSA.

Site I and II corresponds to sock-shaped pockets in subdomain IIA and IIIA of HSA, respectively (5,11). The crystal structure of HSA indicates that these subdomains share a common interface which is stabilized by both hydrophobic and salt bridge interactions (5). This observation led us to the conclusion that site I could possibly communicate with site II through interactions at this interface. In the present study, the hypothesis was tested by obtaining equilibrium dialysis data on WF and DNSA which bind to site I and on IS and DNSS which are site II-ligands. Analysis of the experimental results made it possible to through new light on the potential interactions between site I and II at physiological pH. Furthermore, the influence of the N to B transition on these interactions were examined.

Interaction Between Site I and II Ligands at Physiological pH

Site I is thought to be a wide binding area, consisting of the overlapping binding sites for warfarin and azapropazone (14). Moreover, very recently, results from this laboratory indicated that site I is comprised of at least three subsites which we named Ia, Ib and Ic and which corresponds to the high-affinity binding sites for WF (or acenocoumarol), DNSA and n-butyl p-aminobenzoate, respectively (15). Of these,

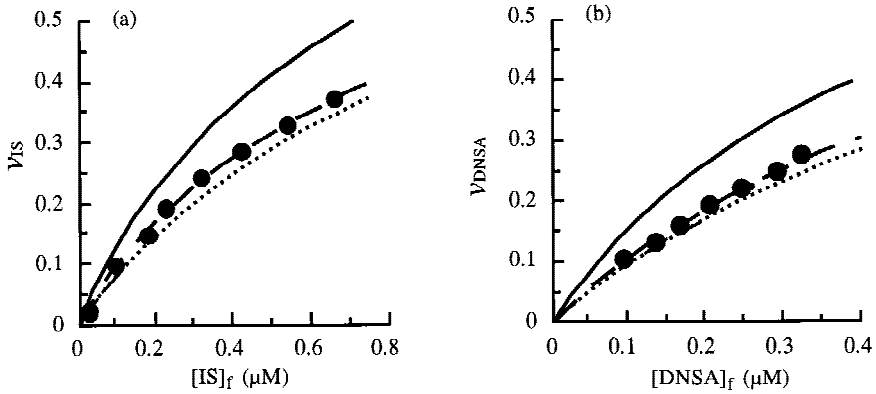

Fig. 2. Binding of IS and DNSA to HSA in the presence of each other at pH 6.5 and 25 °C. (a), Binding of IS (4–16 μM) to HSA (40 μM) in the presence of DNSA (28 μM) (•). (b), Binding of DNSA (6–18 μM) to HSA (40 μM) in the presence of IS (20 μM) (•). The results are means of at least four experiments. (——), Theoretical curves assuming independent binding of the two ligands. (- - - -), Theoretical curves for IS (a) and DNSA (b) binding assuming anti-cooperative interaction between the two ligands (χ =0.12).(…), Theoretical curves for IS (a) and DNSA (b) binding assuming competition between the two ligands for a common binding site.

subsite Ic seems to overlap with subsite Ia (WF site) but it is located apart from subsite Ib (DNSA site). Now, it would be of interest to determine if, at physiological pH, possible site-site interactions exist between site II and all of site I or only between site II and a subsite of site I. Among the subsite probes of site I, IS inhibited only the binding of DNSA. By contrast, it is unlikely that subsite Ia (WF site) communicates with site II under the experimental conditions employed here. The effect of IS on binding of n-butyl p-aminobenzoate was not studied, because this binding site (subsite Ic) is independent of subsite Ib and placed more separately from subsite Ib than the WF binding site (15). The coupling constant obtained from the computed analysis of mutual displacement of IS and DNSA clearly indicated that a reciprocal anti-cooperative interaction exists between these two ligands when bound to HSA (Fig. 1).

A priori, the increase of the free DNSA concentration caused by binding of IS could also be explained by an alternative model, in which the two ligands bind independently to their primary sites but interact, competitively or allosterically, when bound to secondary sites. However, this possibility is readily excluded as an explanation for the decreased binding of DNSA, because small values of r (<0.4) were always used when studying simultaneous ligand binding (Figs. 1–3). Under such conditions, the overwhelming part of both IS and DNSA are bound to their respective primary sites.

Effect of the N to B Transition on the Interaction Between Site I and II

HSA is known to undergo a conformational change between pH 6 and 9, the socalled N to B transition (16–18). At pH 6, HSA essentially exists in the N conformation, whereas at pH 9.0 almost all of the molecules are in the B conformation. Several studies have shown that the N conformation is much more sensitive than the B conformation to the compo-

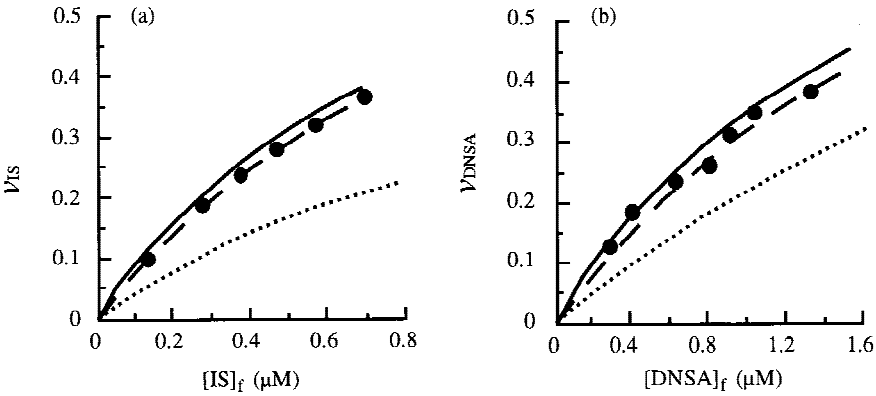

Fig. 3. Binding of IS and DNSA to HSA in the presence of each other at pH 8.5 and 25 °C. (a), Binding of IS (4–16 μM) to HSA (40 μM) in the presence of DNSA (28 μM) (•). (b), Binding of DNSA (6–16 μM) to HSA (40 μM) in the presence of IS (20 μM) (•). The results are means of at least four experiments. (——), Theoretical curves assuming independent binding of the two ligands. (- - - -), Theoretical curves for IS (a) and DNSA (b) binding assuming anti-cooperative interaction between the two ligands (χ = 0.86).(…), Theoretical curves for IS (a) and DNSA (c) binding assuming competition between the two ligands for a common binding site.

sition of the solution in which the albumin is dissolved (17,18). The pH-dependency of the coupling constant, χ, as shown in Fig. 4, can be reasonably well explained by changes in allosteric responses between subsite Ib and site II. In other words, there exists quantitatively a "competitive-like" antagonism between DNSA and the site II-ligand when they bind to the N conformer, while this pairwise interaction becomes quantitatively "independent-like" when binding takes place to the B conformation.

If the allosteric responses between subsite Ib and site II originates from the N to B transition, it can be expected that the N to B transition affects the microenvironmental changes of the binding sites and thereby causes changes in binding of site I and/or II ligands to HSA. Under the present experimental conditions, IS and DNSA exist essentially on the anionic form. Therefore, the above findings cannot be explained by changes in the physicochemical properties of the ligands but must be caused by pH-dependent conformational changes of HSA.

Unlike the N conformer, the three dimensional structure of the B conformer is not known thus far, and, therefore, it is not possible to reveal the precise molecular mechanism of the pH-dependent coupling interaction between site Ib and II. Our previous fluorescence results indicated that co-binding of a site II-ligand (diazepam or ibuprofen) and DNSA, but not subsite Ia or Ic-markers, induced a decrease in solvent accessibility of DNSA in subsite Ib when HSA was isomerized from the N to the B conformation (7). Recently, Bos et al. (19) proposed that, during N to B transition, the albumin molecule changes from a "heart shaped" structure to a more open ellipsoid structure. As mentioned above, an allosteric effect could be transmitted through a common interface of subdomains IIA and IIIA. This means that if the binding subdomains no longer share a common interface due to a conformational change, site interactions will disappear. Consequently, accumulation of uremic toxins and slight decrease of pH may cause increase of the free fraction of drug through either direct interaction (competitive displacement) or indirect interaction (allosteric interaction or site-site interaction) in patients with renal failure. Thus, the findings obtained here will provide useful information for elucidating the complicated mechanism of drug disposition in renal disease state.

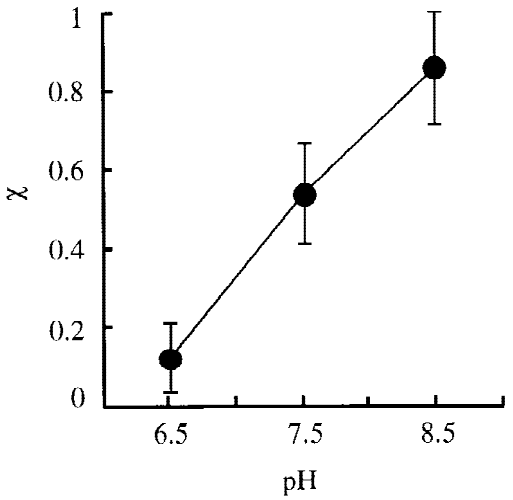

Fig. 4. Effect of pH on the coupling constant (X) for the IS-DNSA interaction. χ-values were determined at *r*-values less than 0.4 in order to minimize ligand binding to low-affinity sites. The results are means ± S.D. for at least four determinations.

REFERENCES

1. W. E. Lindup, S. J. Henderson, and C. E. Barker. Drug binding in renal disease. In F. Belpaire, M. Bogaert, J. P. Tillement, and E. Verbeeck (eds.), *Plasma Binding of Drug and Its Consequence*, Academic Press, Ghent, 1991 pp. 103–120.
2. W. E. Lindup and C. J. Bowmer. Decreased drug binding in uremia: Effect of indoxyl sulfate and other endogenous substances on the binding of drugs to human albumin. *Biochem. Pharmacol.* **31**:319–323 (1982).
3. N. Takamura, T. Maruyama, and M. Otagiri. Effect of uremic toxins and fatty acid on serum protein binding of furosemide: Possible mechanism of the binding effect in uremia. *Clin. Chem.* **43**:2274–2280 (1997).
4. T. Sakai, A. Takadate, and M.Otagiri. Characterization of binding site of uremic toxins on human serum albumin. *Biol. Pharm. Bull.* **18**:1755–1761 (1995).
5. D. C. Carter and J. X. He. Structure of serum albumin. *Adv. Protein Chem.* **45**:153–203 (1994).
6. S. Curry, H. Mandelkow, P. Brick, and N. Franks. Crystal structure of human serum albumin complexed with fatty acid reveals an asymmetric distribution of binding sites. *Nat. Struct. Biol.* **5**: 827–835 (1998).
7. K. Yamasaki, T. Maruyama, K. Yoshimoto, Y. Tsutsumi, R. Narazaki, A. Fukuhara, U. Kragh-Hansen, and M. Otagiri. Interactive binding to the two principal ligand binding sites of human serum albumin: Effect of the neutral-to-base transition. *Biochim. Biophys. Acta* **1432**:313–323 (1999).
8. R. F. Chen. Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.* **242**:173–181 (1967).
9. K. Yamaoka, Y. Tanigawara, T. Nakagawa, and T. Uno. A pharmacokinetic analysis program (MULTI) for microcomputer. *J. Pharmacobio-Dyn.* **4**:879–885 (1981).
10. U. Kragh-Hansen. Relations between high-affinity binding sites of markers for binding regions on albumin. *Biochem. J.* **225**:629–638 (1985).
11. T. Peters, Jr. *All About Albumin: Biochemistry, Genetics, and Medical Applications*, Academic Press, San Diego, California, 1996.
12. U. Kragh-Hansen. Molecular aspects of ligand binding to serum albumin. *Pharmacol. Rev.* **33**:17–53 (1981).
13. R. Brodersen, T. Sjödin, and I. Sjöholm. Independent binding of ligands to human serum albumin. *J. Biol. Chem.* **252**:5067–5072 (1977).
14. K. J. Fehske, W. E. Müller, and U. Wollert. The location of drug binding sites in human serum albumin. *Biochem. Pharmacol.* **30**: 687–692 (1981).
15. K. Yamasaki, T. Maruyama, U. Kragh-Hansen, and M. Otagiri. Characterization of site I on human serum albumin: Concept about the structure of a drug binding site. *Biochim. Biophys. Acta* **1295**:147–157 (1996).
16. J. Wilting, W. F. van der Giesen, L. H. M. Janssen, M. M. Weideman, M. Otagiri, and J. H. Perrin. The effect of albumin conformation on the binding of warfarin to human serum albumin. The dependence of the binding of warfarin to human serum albumin on the hydrogen, calcium and chloride ion concentrations as studied by circular dichroism, fluorescence, and equilibrium dialysis. *J. Biol. Chem.* **255**:3032–3037 (1980).
17. J. Wilting, B. J. Hart, and J. J. de Gier. The role of albumin conformation in the binding of diazepam to human serum albumin. *Biochim. Biophys. Acta* **626**:291–298 (1980).
18. S. Wanwimolruk and D. J. Birkett. The effects of N-B transition of human serum albumin on the specific drug-binding sites. *Biochim. Biophys. Acta* **709**:247–255 (1982).
19. O. J. M. Bos, J. F. A. Labro, M. J. E. Fischer, J. Wilting, and L. H. M. Janssen. The molecular mechanism of the neutral-to-base transition of human serum albumin. An acid/base titration and proton nuclear magnetic resonance studies on a large peptic and a large tryptic fragment of albumin. *J. Biol. Chem.* **264**:953–959 (1989).