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Biochemical and Biophysical Behavior of Plasma Albumin. III. Effects of Several Uremic Toxins on Normal Plasma Albumin

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The effects of some uremic toxins on the structure of normal human plasma albumin were investigated in order to cast light on the state of plasma albumin of patients with chronic renal failure. Two different ion exchange columns were prepared to isolate and purify albumin, and β-indoleacetic acid (IAA), β-indolebutyric acid (IBA), guanidinosuccinic acid (GSA) or urea as a uremic toxin was added to the normal plasma albumin. α-Helix contents as estimated from the circular dichroism spectra were found to be little affected by uremic toxins. The effect of uremic toxins on the binding of a fluorescent probe, 1-anilino-8-naphthalene sulfonate (ANS), to albumin was examined by calculating the binding parameters, the association constant and the number of binding sites. All uremic toxins decreased the association constants. GSA and urea decreased the number of binding sites, while IAA and IBA increased them. These effects increased as the concentrations of toxins added were increased, especially with IAA and IBA.

Keywords——chronic renal failure; albumin; uremic toxin; circular dichroism; α-helix content; fluorescent probe technique; hydrophobic region

Plasma from patients with chronic renal failure has a decreased ability to bind many acidic drugs;[1–3] this effect was first observed for some sulfonamides.[4] The decreased binding in the patient's plasma is restored to the normal level by therapeutic hemodialysis. Thus, it can be hypothesized that the binding of certain drugs to the plasma protein is impaired in patients with chronic renal failure due to an accumulation of some endogenous substances in the plasma which are normally excreted in urine.[5] Such endogenous substances are called uremic toxins.[6,7]

In the present study, the effects of some uremic toxins on the conformation of the albumin molecule were investigated in order to cast light on the state of plasma albumin of patients with chronic renal failure. β-Indoleacetic acid (IAA), β-indolebutyric acid (IBA) and guanidinosuccinic acid (GSA) were chosen as uremic toxins because of their high inhibitory effect on protein binding,[8] and urea because of its toxicity at high concentration. Circular dichroism (CD) spectra were used to evaluate the conformational features of albumin in the presence and absence of uremic toxins and α-helix contents were calculated. We also used the fluorescent probe technique as a convenient method for estimating the nature of binding and the number of binding sites.

Experimental

Materials——Normal human plasma was supplied by the Japanese Red Cross Hokkaido Blood Center, stored in a deep-freeze at about −70°C and used within one week. IAA and IBA were obtained from Tokyo Kasei Co., Ltd., Tokyo, and GSA was from Sigma Chemical Co., U.S.A. The fluorescent probe, 1-anilino-8-naphthalene sulfonate (ANS), was purchased as the sodium salt from Kanto Chemical Co., Inc., Tokyo. All other chemicals of analytical grade were obtained from the usual commercial sources, and were used without further purification. Redistilled water was used for measuring fluorescence intensity.

Isolation and Purification of Albumin——Plasma albumin was isolated as reported in the preceding paper.[9] Cellulose acetate membrane electrophoresis showed the product to be 98% pure. This albumin

was appropriately diluted with pH 7.4 phosphate buffer and the concentration of the solution was determined spectrophotometrically at 280 nm using a Hitachi spectrophotometer (Model 100—200). Each sample solution with a uremic toxin was prepared as follows: a desired concentration and volume of IAA, IBA, GSA or urea were added to a certain concentration of albumin solution and the mixture was stirred moderately.

CD Measurements——CD measurements for each sample solution were performed with a Jasco J-20 spectropolarimeter (Japan Spectroscopic Co., Tokyo). A jacketed cylindrical cell with an optical path length of 1 mm was used, and measurements were made at 23±2°C. The concentration of albumin in each sample solution ranged from 0.015 to 0.15% in 1/15 M phosphate buffer, pH 7.4. The results of CD measurements are expressed in terms of molar ellipticity ($\{\theta\}$ in degrees $\times$ cm^2 $\times$ dmol^{-1}) which is defined according to equation (1):

$$\{\theta\} = \frac{\theta \cdot M}{c \cdot l \cdot 10} \tag{1}$$

where θ is the observed ellipticity in degrees, M is the molecular weight, c the concentration of albumin solution in g/ml and l the path length of the cell in cm. A molecular weight of 66500 was assumed for albumin.[10] The α-helix contents were calculated by the method of Greenfield and Fasman,[11] using equation (2).

$$\alpha\text{-helix content} = \frac{-\{\theta\} - 4000}{33000 - 4000} \tag{2}$$

Fluorescence Measurements——Fluorescence measurements were made with a Shimadzu RF-510 fluorescence spectrophotometer. The fluorescence intensity of the protein-ANS complex as a function of probe concentration was measured at an appropriate protein concentration (0.015% and 0.30%) in pH 7.4 phosphate buffer. Two ml of protein solution was titrated by successive additions of 2 μl of 1×10^{-3} M ANS in the phosphate buffer. Fluorescence titrations were performed manually with a microsyringe (Type 10A RN-GP, S.G.E. PTY Ltd., Australia). The excitation and emission wavelengths were 375 and 465 nm, respectively. All experiments were done at room temperature (approx. 23°C). Under the conditions used in these experiments, fluorescence arising from ANS in the phosphate buffer was negligible. The enhancement of the fluorescence of the probe upon addition to albumin solution was used to calculate the binding parameters for the probe. The fraction of probe bound, X, was calculated using the following equation (3).

$$X = (I_0 - I_f)/(I_b - I_f) \tag{3}$$

Where I_0 and I_f refer to the fluorescence intensities of a given concentration of probe in a solution of low protein concentration and in a solution without protein, respectively; and I_b refers to the fluorescence intensity of the same concentration of probe in a solution of high protein concentration. After the value X is found for each point along the titration curve, the Scatchard equation (4)[12] may be applied to calculate the association constant of the probe;

$$\bar{V}/A = nK_a - \bar{V}K_a \tag{4}$$

where $\bar{V}$ is the number of moles of bound probe per mole of protein, A is the concentration of free probe, n is the number of binding sites on the protein molecule, and K_a is the association constant of the probe to the protein. The value of $\bar{V}$ is determined by multiplying the value for X by the ratio of the total probe concentration to the total protein concentration in solution. The data were analyzed by the non-linear least-squares method.

Results and Discussion

In order to investigate whether or not uremic toxins alter the conformation or structural arrangement of the albumin molecule, the uremic toxins listed in Table I were added to normal plasma albumin and CD spectra was measured. The spectra were very similar in form and magnitude, and were typical of α-helical polypeptides and proteins. Mean α-helix contents calculated by means of equation (2) are shown in Table I. The addition of uremic toxins to normal plasma resulted in slightly lower or unchanged α-helix contents compared to control levels. This result suggests that these uremic toxins at 3.33×10^{-5} M and 3.33×10^{-4} M[13] produced only minor changes in the helical structure of normal plasma albumin.

A decreased ability of plasma albumin to bind certain drugs in patients with chronic renal failure could be due to a conformational change in the albumin molecule causing a decrease in the number of binding sites in the protein, or to a decrease in the association constants of the sites. These possibilities were examined by using ANS as a marker for binding capacity. Fluorescence data were analyzed by using equation (4), and the results are shown in Figs. 1

Table I. Effects of Uremic Toxins on α-Helix Contents of Albumin

Added material	Concentration of added material (M)	α-Helix content[a] (%)
None	—	45.5±1.8
IAA	3.33×10^{-5}	44.1±1.4
	3.33×10^{-4}	45.2±2.1
IBA	3.33×10^{-5}	42.4±0.7
	3.33×10^{-4}	42.9±2.6
GSA	1.67×10^{-5}	43.1±1.2
	1.67×10^{-4}	45.6±1.1
Urea	3.33×10^{-4}	43.8±0.9
	3.33×10^{-3}	41.8±1.7

a) Mean ± s.e. (n=5).

and 2. When the Scatchard plot is curved as shown in Fig. 1 (without uremic toxin or with GSA and urea), two kinds of binding sites can be assumed. On the other hand, IAA or IBA gave a straight line as shown in Fig. 2, which suggests that only one binding site was present. The binding parameters obtained from Figs. 1 and 2 are summarized in Tables II and III. As shown in Table II, the presence of lower concentrations of GSA and urea decreased the values of K_1 and higher concentrations decreased both K_1 and K_2. The higher concentrations of GSA and urea gave smaller K_1 than the lower concentrations. The results also show that GSA and urea decreased the values of n_1 and n_2, except at the higher concentration of urea. IAA and IBA gave only one binding site and changed the values of both K_2 and n_2 (Table III). The values of K_2 were decreased, and the values of n_2 were increased, and the effects were more pronounced at the higher concentrations of the uremic toxins. It seems that no interaction occurs between ANS and each uremic toxin, as changing the concentration of ANS solution

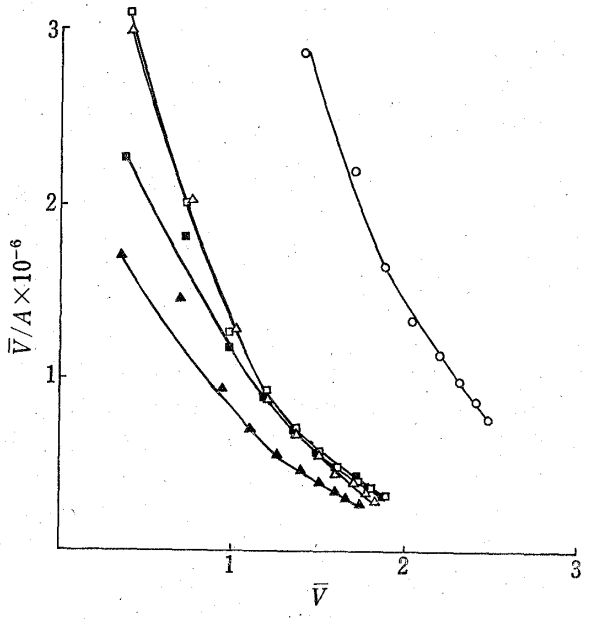

Fig. 1. Scatchard Plots of ANS binding to Albumin with or without a Uremic Toxin (Average of 3 experimental runs)

○, without a uremic toxin; △, with GSA (1.67×10^{-5} M); ▲, with GSA (1.67×10^{-4} M); □, with urea (3.33×10^{-4} M); ■, with urea (3.33×10^{-3} M).

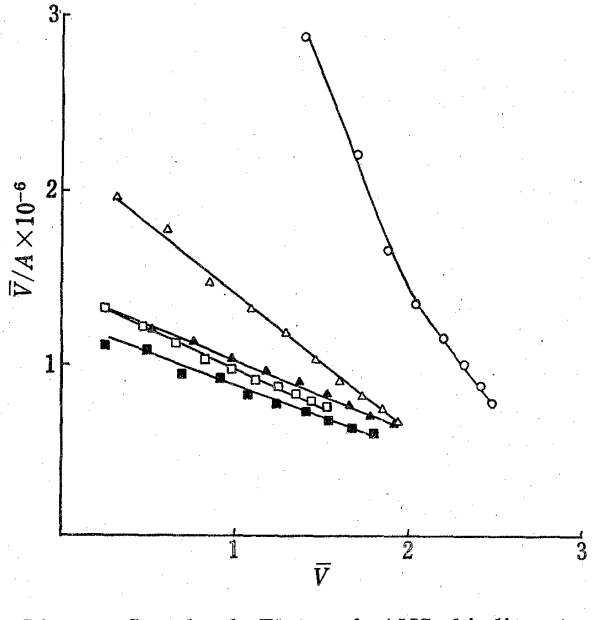

Fig. 2. Scatchard Plots of ANS binding to Albumin with or without a Uremic Toxin (Average of 3 experimental runs)

○, without a uremic toxin; △, with IAA (3.33×10^{-5} M); ▲, with IAA (3.33×10^{-4} M); □, with IBA (3.33×10^{-5} M); ■, with IBA (3.33×10^{-4} M).

Table II. Binding Parameters of ANS with Plasma Albumin with or without a Uremic Toxin (Average of 3 experimental runs)

Uremic toxin	Concentration of uremic toxin (M)	$K_1 \times 10^6$ (M^{-1})	$K_2 \times 10^6$ (M^{-1})	n_1	n_2
None		3.42	0.711	1.61	1.41
GSA	1.67×10^{-5}	2.63	0.826	1.24	0.955
	1.67×10^{-4}	1.35	0.603	1.27	0.926
Urea	3.33×10^{-4}	2.72	0.730	1.21	1.11
	3.33×10^{-3}	1.67	0.699	1.92	1.00

Table III. Binding Parameters of ANS with Plasma Albumin with or without a Uremic Toxin (Average of 3 experimental runs)

Uremic toxin	Concentration of uremic toxin (M)	$K_1 \times 10^6$ (M^{-1})	$K_2 \times 10^6$ (M^{-1})	n_1	n_2
None		3.42	0.711	1.61	1.41
IAA	3.33×10^{-5}		0.375		2.83
	3.33×10^{-4}		0.206		3.51
IBA	3.33×10^{-5}		0.222		3.19
	3.33×10^{-4}		0.174		3.49

had no effect on the UV spectrum of each uremic toxin solution. These results suggest that certain uremic toxins cause a decrease in the number of binding sites on the albumin molecule and/or in the association constants. Low concentrations of GSA and urea seem to inhibit the binding of ANS to albumin at one of the binding sites on the albumin molecules, as the values of K_1 are decreased and those of K_2 are not. High concentrations of GSA and urea seem to inhibit the binding at both binding sites on albumin molecules. IAA and IBA decrease the values of K_2 considerably and apparently only one kind of binding site exists; this may result from the high binding affinities of IAA and IBA to albumin. IAA and IBA may block the first binding site on albumin molecules for ANS. It has been reported that ANS binds to albumin at its hydrophobic region,[14] so uremic toxins may affect ablumin at this region. The smaller values of n_1 and n_2 when GSA or urea was added and the larger values of n_2 when IAA or IBA was added may reflect the extent of possible constriction or expansion of the steric structure in the hydrophobic region induced by the uremic toxins. Brown[15] found that most hydrophobic residues were located between the "helices" and inside the trough, whereas the great majority of polar residues was on the outer wall of the structure. The uremic toxins have little influence on the α-helical structure of albumin molecules, but have a significant effect on the structure of the hydrophobic region; thus, the changes in α-helix contents may be induced secondarily by the constriction or expansion of the hydrophobic region.

In this study, we considered only the conformational changes of albumin molecules. The possibility of a displacing effect of ANS on the bound uremic toxins remains to be elucidated.

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