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Interaction of the Fluorescent Probe 7-Anilino-4-methylcoumarin-3-acetic Acid with α-Globulin

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The fluorescence characteristics of 7-anilino-4-methylcoumarin-3-acetic acid (AMCA), which may be useful as a fluorescent probe in protein binding investigations, were examined in various solvents and α-globulin solution. The fluorescence of AMCA was significantly enhanced in less-polar solvents and in the presence of α-globulin with a shift of the emission maximum to shorter wavelength. Scatchard plots and continuous variation plots indicated that one molecule of AMCA binds to α-globulin. AMCA was applied as a probe in binding studies of some estrogens and corticoids with α-globulin. The spectral changes of the AMCA–α-globulin system in the presence of steroids indicated that estrogens (α- and β-estradiol, estriol) competitively displace AMCA bound to α-globulin but corticoids (cortisone, hydrocortisone) do not. The binding parameters of estrogens for α-globulin were also estimated. The present data suggest that AMCA is useful as a fluorescent probe for assessing the binding of steroids, including estrogens, to α-globulin.

Keywords——7-anilino-4-methylcoumarin-3-acetic acid; α-globulin; protein-binding fluorescent probe; 7-anilino-4-methylcoumarin-3-acetic acid–α-globulin binding; estrogen–α-globulin binding parameter; fluorescence quantum yield

The fluorescent probe technique has been employed extensively in investigations of plasma protein binding properties. Considerable interest has been focused on serum albumin as a carrier for drugs and other small molecules. We recently reported a new fluorescent probe, 7-anilinocoumarin-4-acetic acid (ACA), for the third drug binding site (the so-called digitoxin site) on human serum albumin.[1] However, at the present stage of protein binding studies, little is known about the binding features of globulins among plasma proteins.[2]

Thus, this paper deals with the interaction between human α-globulin and 7-anilino-4-methylcoumarin-3-acetic acid (AMCA) to investigate the possible utility of AMCA as a probe in drug-protein binding studies.

Experimental

Materials——Human α-globulin (Fraction IV, Lot. No. 29) was obtained from Miles Laboratories, Inc., Elkhart Ind. AMCA was that reported previously, and was recrystallized from EtOH to give a single spot on a silica gel thin layer chromatogram. Cortisone, α- and β-estradiols, hydrocortisone (Sigma Co.), estriol and estrone (Merck Co.) were purchased from Wako Pure Chemical Ind. Ltd., Osaka, and Tokyo Kasei Kogyo Co., Ltd., Tokyo respectively. All of the organic solvents used for the spectral measurements were of spectrograde, and deionized and distilled water was used throughout.

Apparatus and Method——Fluorescence spectra were measured with a Hitachi 650-60 fluorescence spectrophotometer. Fluorescence quantum yields were determined according to the method of Parker and Rees,[3] and quinine sulfate in 1 N H_2SO_4 was used as a standard. Gel permeation chromatography for the estimation of α-globulin molecular weight (159000) was carried out on a Shodex RI SE-31 apparatus equipped with a ultraviolet (UV) detector (250 nm) and a Shodex column (AQP^{9k}G40A-R-404) at room temperature. A 1/30 M phosphate buffer–1/5 M

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sodium sulfate (pH 6.5) system was used as the eluent at a flow rate of 0.5 ml/min. All α-globulin and AMCA solutions were prepared in 1/10 M phosphate buffer of pH 7.4 (sodium phosphate dibasic and potassium phosphate monobasic) at $25 \pm 1\,^{\circ}C$.

Fluorometric Titrations: α-Globulin solution at an appropriate concentration was titrated by successive additions of a solution of AMCA (to give a final concentration of 1.0×10^{-5} M) and the fluorescence intensity was measured (excitation at 380 nm and emission at 468 nm).

Data Treatment: The fraction of AMCA bound, X, is usually determined by using equation 1, where F_p and F_0 are the fluorescence intensities of a given concentration of AMCA in a solution of low α-globulin concentration and in a solution without α-globulin, and

$$X = \frac{F_p - F_o}{F_b - F_o} \qquad (1)$$

F_b is the fluorescence of the same concentration of fully bound AMCA. To determine the values of F_b for a given concentration of AMCA, fluorescence titrations were carried out at several α-globulin concentrations. F_b is taken to be the fluorescence intensity of the substrate in the presence of excess α-globulin. After values for the fraction of bound AMCA had been found for all points along the titration curve, the results were plotted according to the Scatchard equation:[4]

$$r/D_f = nK_a - rK_a \qquad (2)$$

where r is the number of mol of AMCA bound per mol of protein, n is the number of binding sites, K_a is the binding constant and D_f is the concentration of free AMCA. The binding constants of competitive compounds were calculated by using the equation of Klotz *et al.*:[5]

$$K_b = \frac{n\,P_t\,K_a\,D_f - K_a\,D_f\,D_b - D_b}{B_t\,K_a\,D_f - n\,P_t\,K_a\,D_f + K_a\,D_f\,D_b + D_b} \times \frac{K_a\,D_f}{D_b}$$

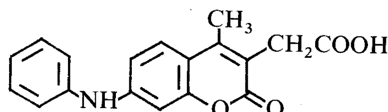
where K_a and K_b are the binding constants for the probe (AMCA) and the competitor (estrogen), D_f is the concentration of free probe, D_b is the concentration of bound probe, P_t is the total concentration of protein (α-globulin), B_t is the total concenration of competitor, and n is the number of binding sites involved.

Results and Discussion

Fluorescence Characteristics of AMCA

When the fluorescence quantum yields or emission maxima of certain organic compounds are related to the polarity of the environment around the fluorescent species, these compounds can serve as probes in protein binding studies. The fluorescence spectrum of AMCA was first measured in a variety of solvents, and spectral data are summarized in Table I. The fluorescence spectra of AMCA in water, ethanol and cyclohexane reported in a previous paper[1] were remeasured under the present conditions because the spectrophotom-

TABLE I. Fluorescence Spectral Data for 7-Anilino-4-methylcoumarin-3-acetic Acid (AMCA) in Various Solvents



Solvent	$F\lambda_{max}$ nm (Quantum yield)[a]	Solvent	$F\lambda_{max}$ nm (Quantum yield)[a]
Water[b]	—	1-Octanol	465 (0.171)
Acetonitrile	484 (0.008)	Chloroform	455 (0.325)
Methanol	477 (0.004)	Benzene	431 (0.467)
Ethanol	468 (0.013)	Cyclohexane	419 (0.474)
n-Butanol	466 (0.052)		

a) Excitation wavelength: 380 nm. *b*) Phosphate buffer (pH 7.4).

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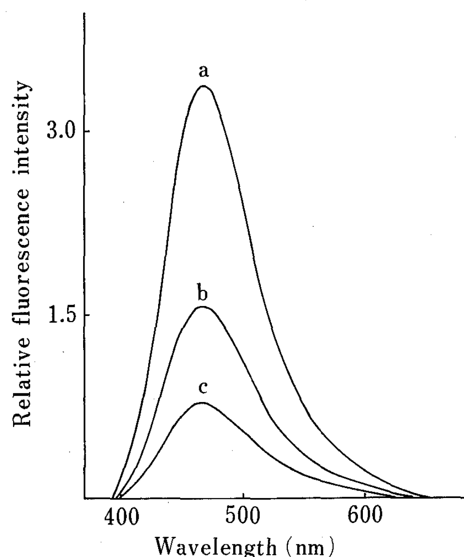

Fig. 1. Fluorescence Spectra of AMCA (1.0×10^{-6} M) in the Presence of α-Globulin

α-Globulin concentration: a, 5.0×10^{-6} M; b, 2.0×10^{-6} M; c, 1.0×10^{-6} M.

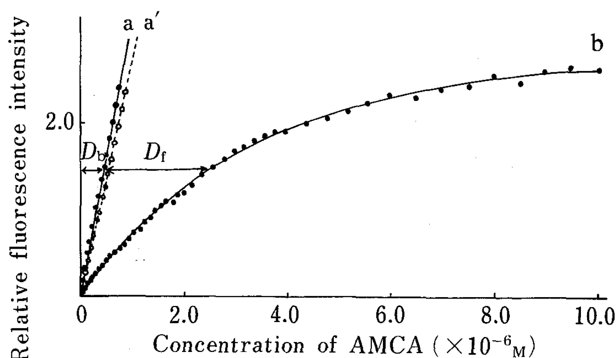

Fig. 2. Relative Fluorescence Intensity in the AMCA–α-Globulin Interaction as a Function of the Concentration of AMCA

α-Globulin concentration: a′, 1.5×10^{-5} and 2.0×10^{-5} M; b, 1.5×10^{-6} M. The straight line a was obtained after correcting the fluorescence intensities in curve a′ for the absorbance effect.

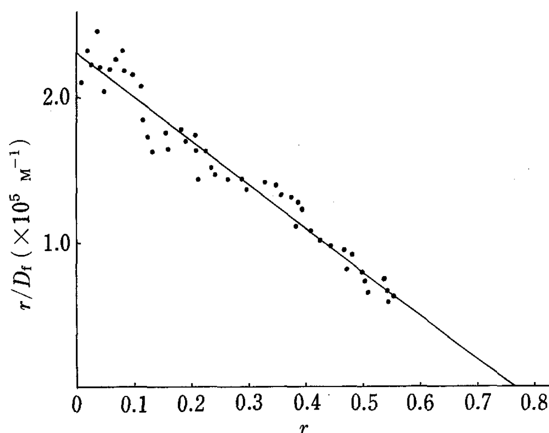

Fig. 3. Scatchard Plots of AMCA–α-Globulin Interaction

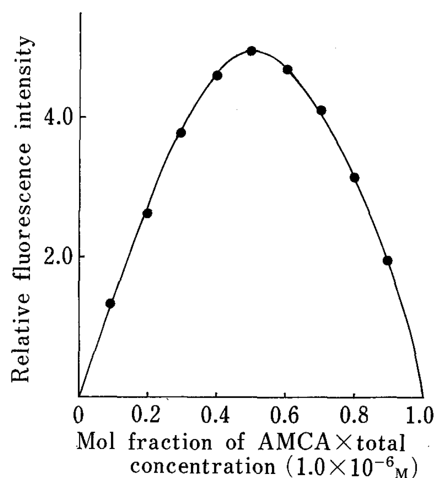

Fig. 4. Job's Plot of Relative Fluorescence Intensity for the AMCA–α-Globulin Interaction

The total concentration of [α-Globulin]+[AMCA] was kept at 1.0×10^{-6} M.

eters and the exciting wavelength were different from those used before. As shown in Table I, the fluorescence of AMCA varied significantly with the polarity of solvents; for example, AMCA was practically nonfluorescent in aqueous solution but was highly fluorescent in cyclohexane (quantum yield, 0.474), and hence AMCA seems to be suitable for use as a hydrophobic probe. Furthermore, the fluorescence behavior of AMCA in the presence of α-globulin in pH 7.4 phosphate buffer was also examined and the fluorescence spectra are shown in Fig. 1. The addition of α-globulin resulted in a large increase in the fluorescence intensity of AMCA accompanied by a slight blue shift of the emission maximum. These observations suggest that AMCA should be useful as a probe for protein binding.

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Interaction between AMCA and α-Globulin

To estimate the binding parameters for AMCA–α-globulin interaction, fluorometric titration was done by altering AMCA concentration as shown in Fig. 2. Fluorescence intensities for two titrations with high α-globulin concentrations (1.5×10^{-5} and 2.0×10^{-5} M) were identical, suggesting that AMCA added was fully bound at both protein concentrations (broken line a′). For this treatment to be valid, the fluorescence intensity of the bound AMCA must be a linear function of concentration. This is the case only when the absorbance of the complex at the exciting wavelength is low. A correction for this absorbance can be made by the method of Naik *et al.*[6] and was made for all the data when the absorbance at 380 nm was greater than 0.02. A straight line (line a in Fig. 2) was obtained, after correcting the observed fluorescence intensities for the absorbance effect (the second term in the correction equation was considered). When the titration was carried out at low concentration, AMCA was only partially bound (curve b). The plateau in the titration curve obtained at low protein concentration indicates saturation of the α-globulin binding site. The linearity of Scatchard plots for the AMCA–α-globulin system obtained from the titration curves in Fig. 2 indicates that AMCA binds to one class of sites on α-globulin (Fig. 3). The binding constant, K_a and the n value estimated from Scatchard plots were 3.01×10^5 M^{-1} and 0.76, respectively. To check the maximum number of binding sites, a Job's plot[7] was also prepared for the AMCA–α-globulin system by keeping the total concentration of AMCA and α-globulin at 1.0×10^{-6} M (Fig. 4). The inflection point for this plot is near 0.5, the value expected for 1:1 complex formation. The n value estimated from the Scatchard plot was in fair agreement with that from Job's plot. Slight differences between the n values obtained by the two methods may have arisen from the difference of data treatment. Therefore, it is reasonable to conclude that the maximum number of binding sites contributing to the fluorescence in the AMCA–α-globulin interaction is one.

AMCA as a Fluorescent Probe for Steroid Binding to α-Globulin

It is known that certain steroids such as corticoids and progesterone bind not only to albumin[8] but also to globulins.[9] In order to establish the utility of AMCA in protein binding studies, the effects of the addition of various steroids on the fluorescence of the AMCA–α-

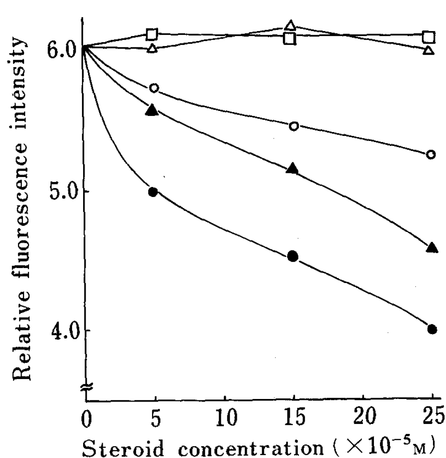

Fig. 5. Effects of Steroids on the Fluorescence Intensity of AMCA (5.0×10^{-6} M) in the Presence of α-Globulin (5.0×10^{-6} M)

○, α-estradiol; ●, β-estradiol; ▲, estriol; △, cortisone; □, hydrocortisone.

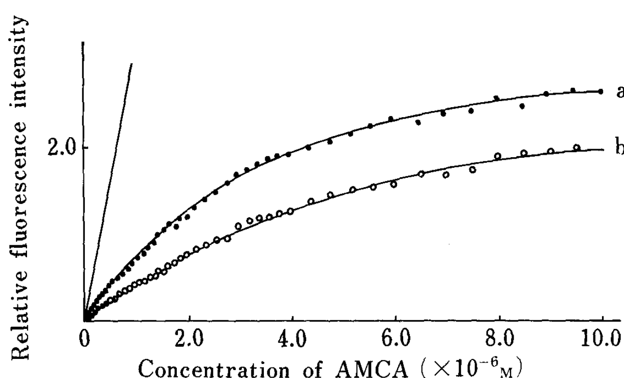

Fig. 6. Relative Fluorescence Intensity in the AMCA–α-Globulin Interaction as a Function of the Concentration of AMCA in the Presence of β-Estradiol

α-Globulin concentration, 1.5×10^{-6} M; β-estradiol concentration, 7.5×10^{-5} M. Curve a, in the absence of β-estradiol; curve b, in the presence of β-estradiol.

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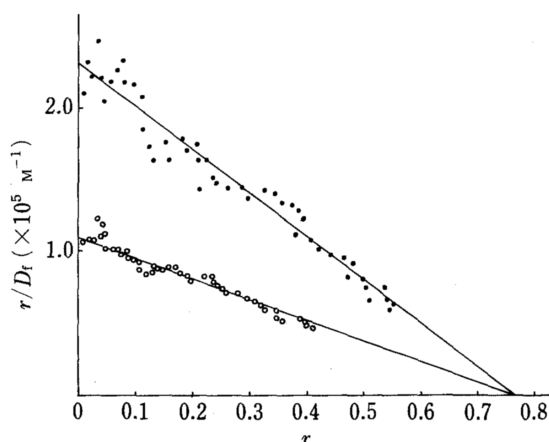

Fig. 7. Scatchard Plots of AMCA–α-Globulin Interaction

●, in the absence of steroid; ○, in the presence of β-estradiol (7.5×10^{-5} M).

TABLE II. Binding Parameters for Estrogens to α-Globulin

Compound	K_b (M^{-1})	n
α-Estradiol	1.45×10^5	0.76
β-Estradiol	1.74×10^5	0.76
Estriol	1.79×10^5	0.76

globulin system were examined. As shown in Fig. 5, the presence of estrogens such as α- and β-estradiols and estriol markedly quenched this fluorescence, suggesting that these estrogens competitively displace AMCA bound to α-globulin. Similar experiments were also tried for estrone but no clear result was obtained because of the limitation of solubility in water. In contrast, no significant change in the fluorescence was observed in the presence of corticoids such as cortisone and hydrocortisone. This suggests that there may be no binding sites for corticoids, that they may bind at other sites that do not affect the fluorescence of AMCA, and that the binding forces of corticoids may be too weak to displace AMCA bound to α-globulin. Further studies employing additional fluorescent probes or other techniques such as equilibrium dialysis will be necessary for the elucidation of the binding (if any) of corticoids.

In this work, the binding parameters for estrogen–α-globulin interactions were estimated by the use of AMCA as a fluorescent probe. Figure 6 shows the fluorometric titration curves for the AMCA–α-globulin system in the presence (line b) and absence (line a) of β-estradiol. As shown in Fig. 6, curve b was below curve a, being consistent with the results presented in Fig. 5. Scatchard plots prepared on the basis of the results of Fig. 6 were linear (Fig. 7) and the binding parameters for β-estradiol–α-globulin estimated in the usual way are summarized together with the results for other estrogens in Table II. The binding constants, K_b, for the estrogens were all quite similar, indicating that the estrogens have approximately the same affinity for α-globulin.

Thus, the binding parameters for some estrogens at low protein concentration could be readily estimated by the use of AMCA as a fluorescent probe, suggesting that AMCA is useful as a probe for the binding of steroids, including estrogens, to α-globulin. This method, however, is only effective for drugs which compete with AMCA for binding to α-globulin. Therefore, further development of different types of fluorescent probes will be required for the recognition of specific drug-binding sites on α-globulin.

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