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INTERACTION OF POLYELECTROLYTES WITH ALBUMIN USING FLUORESCENCE MEASUREMENT

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ABSTRACT

Because proteins are polyampholytes, they can interact with polyelectrolytes, mainly through an electrostatic force. In this report we discuss the interaction of serum albumin, which is a major component of serum proteins, with synthetic polyelectrolytes using fluorescent measurement. Polycations interact with anionic amino acids in a relatively random fashion only above the isoelectric point of albumin. On the other hand, polyanions interact selectively with a specific site of albumin, the drug binding site II in which basic amino acids are localized.

INTRODUCTION

In order to design biomedical polymers, we need fundamental investigations of the interaction between biocomponents (proteins, polysaccharides, cells, tissues, and so on) and synthetic polymers. Moreover, we must pay attention to the important role of macromolecular assemblies in in-vivo bioreactions. From these points of view, it is very interesting and important to clarify the mechanism of the interaction between proteins and synthetic macromolecules.

Oppositely charged polyelectrolytes interact with each other to form polyelectrolyte complexes (PEC) [1–5]. Because proteins are polyampholytes, there have been many reports about the PEC formation of proteins with synthetic polyelectro-

lytes [6–10] in the basic study of these interactions. These reports offer important information on biofunctionality in vivo as well as on a wide range of applications, e.g., bioseparator, immobilization of enzymes, artificial organs, polymer drugs, and so on.

We previously reported on the mechanism of PEC formation by using fluorescence measurements [11–13] in anthracene-loaded artificial polymer systems. On the other hand, the fluorescence of tryptophan (Trp) residues in proteins has been widely used as a probe of the conformational changes of the proteins [14–16]. Therefore, in this article the spectral changes in fluorescence, especially the emission maximum (λ_{em}), are used as parameters for the conformational change of proteins induced by the formation of PEC with synthetic polyelectrolytes.

Serum albumin is a major component of serum proteins and shows various functions in vivo, e.g., control of osmotic pressure, transport and storage of nutriments and drugs, and so on. Moreover, it plays an important role in the interaction between a biomedical polymer surface and biocomponents. The first structures and the conformational changes of albumin have been clarified [17–19], especially for human serum albumin (HSA) and bovine serum albumin (BSA).

We will discuss the interaction of artificial polyelectrolytes with albumin in an aqueous solution as determined by fluorescence measurements.

EXPERIMENTAL

Materials

9-Anthrylmethylmethacrylate (AMMA) and the copolymers of AMMA with methacrylic acid (PMA-A) and sodium 2-acrylamido-2-methylpropanesulfonate (PAMPS-A) were synthesized as reported previously [10]. *N*-Methacryloyl-*α*-*d*,*l*-alanine (NMA) was synthesized by the previously used method [20]. The copolymer of NMA and AMMA (PNMA-A) was obtained as follows: 5×10^{-5} mol AMMA and 2×10^{-2} mol NMA were dissolved in 20 mL *N*,*N*-dimethylformamide (DMF) in the presence of 2,2′-azobis(isobutyronitrile) (1 mol% of the total monomer concentration) as the initiator. The polymerization was achieved in a vacuum-sealed tube at 60°C for 3.5 hours. The resulting polymer was precipitated by an excess of ethyl acetate and further purified by reprecipitation twice from DMF into ethyl acetate. The polymer was then dissolved in water and the solution was dialysed against pure water and finally lyophilized: conversion = 21%, mol fraction of AMMA in the copolymer = 0.33 mol%. The homopolymer of NMA (PNMA) was prepared in the same way without AMMA: conversion = 17%. 3-Acrylamidopropyltrimethylammonium chloride (QP) and 2-acryloylethyltrimethylammonium chloride (QE) were provided by Kojin Co. and used without further purification. The copolymers of AMMA with QP (PQP-A) and QE (PQE-A) were synthesized in the same way as mentioned above: conversion and AMMA content of PQP-A were 24% and 0.56 mol%, respectively, and those of PQE-A were 12% and 0.21 mol%. The homopolymers of QP (PQP) and QE (PQE) were prepared in the same way without AMMA: the conversions of PQP and PQE were 36 and 45%, respectively. Poly[(dimethyliminio)trimethylene(dimethyliminio)methylene-1,4-phenylenemethylene dichlorides] (3X), poly(vinylbenzyltrimethylammonium chloride) (PVBMA), poly(allyltrimethylammonium chloride) (QPAl.Am), poly(methacrylic acid) (PMA),

poly(sodium styrenesulfonate) (PSS), poly(sodium 2-acrylamido-2-methylpropanesulphonate) (PAMPS) were reported previously [10]. The formula and properties of these polyelectrolytes are summarized in Fig. 1 and Table 1.

Bovine serum albumin (BSA) and human serum albumin (HSA) were purchased from Wako Chemical Co. (biochemical grade) and Sigma Chemical Co. (Fraction V), respectively, and used without further purification.

3-(α-Acetonylbenzyl)-4-hydroxycoumarin (warfarin; War, Sigma) and dansyl-L-proline free acid (DNP, Sigma) were purchased and used without further purification.

Measurements

Aqueous solutions of the polyelectrolytes shown in Fig. 1 (final concentration = 10^{-4} mol of ionic sites/L, pH 7.4) were mixed with the aqueous solutions of BSA (final concentration = 4×10^{-2} g/L) or HSA (final concentration = 8×10^{-2} g/L) and then the pH of the solution was changed by adding aqueous solutions of HCl or NaOH (0.1 N).

The fluorescence spectra of tryptophan (Trp) in albumin or anthryl groups (Anth) in those polyelectrolytes were then measured by a Shimadzu RF-5000 spec-

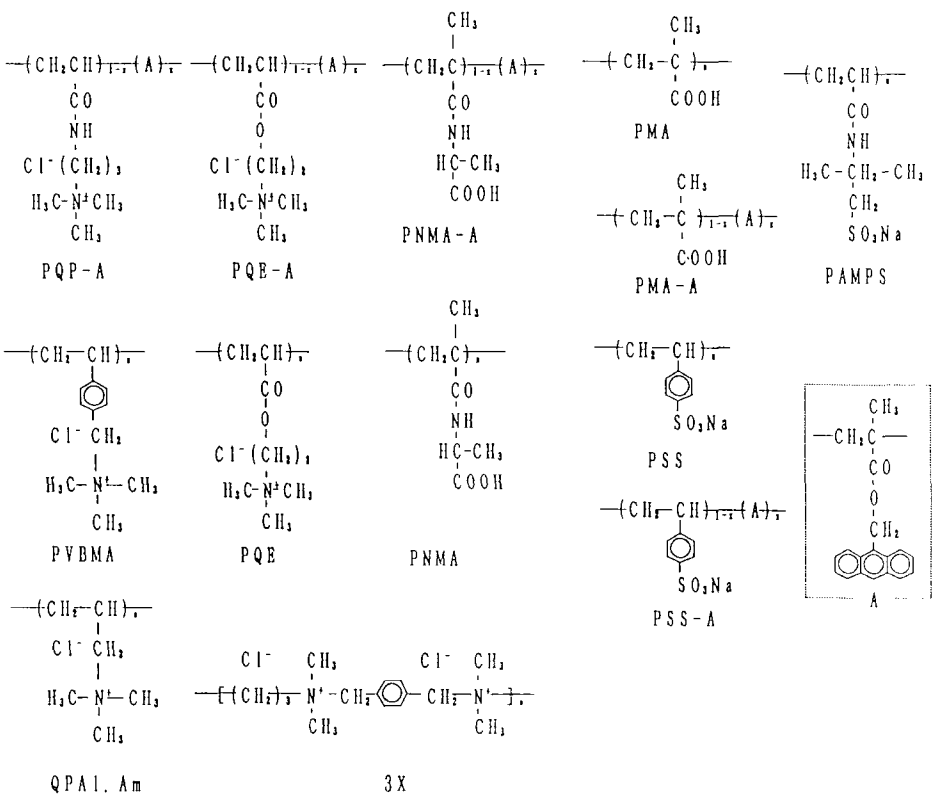

FIG. 1. Chemical formulas of artificial polyelectrolytes.

TABLE 1. Characteristics of Polyelectrolytes

Abbreviation	η_{sp}/c,[a] dL/g	Abbreviation	Anthryl group content,[b] mol%	η_{sp}/c,[a] dL/g
PMA	0.35	PMA-A	0.40	0.087
PSS	0.42	PSS-A	0.48	0.26
PNMA	0.18	PNMA-A	0.25	0.33
PAMPS	0.48			
PQE	0.12	PQE-A	0.21	0.15
		PQP-A	0.56	0.18
PVBMA	0.12			
QPAl.Am	0.34			
3X	0.23			

[a]Reduced viscosity at 0.5 g/dL in 0.2 M of aqueous KCl at 30°C.

[b]The mole fraction of the anthryl group in the copolymer (mol%) was determined by the optical density of the anthryl residue (ϵ = 10,600 $M^{-1} \cdot cm^{-1}$ at 368 nm) in methanol.

trophotometer at room temperature. The excitation wavelengths were 280 nm for Trp and energy transfer systems and 365 nm for Anth, respectively.

Aqueous solutions (pH 7.4) of BSA or HSA at the same concentration as mentioned above were mixed with those of War or DNP (final concentration = 3×10^{-5} mol/L) and incubated for about 1 hour at room temperature. Then aqueous solutions (pH 7.4) of the polyelectrolytes at the same concentration were added to the albumin solutions. The fluorescences of these solutions were measured. The excitation wavelength was 280 nm.

RESULTS AND DISCUSSION

It is well known that the fluorescence of tryptophan (Trp) of albumin (Alb) varies with its conformational change, e.g., the blue shift of the emission maximum (λ_{em}) and the quenching. As shown in Fig. 2, the fluorescence intensity at λ_{em} of Trp in BSA was reduced (quenching) and the λ_{em} was blue-shifted in the presence of polyelectrolytes at pH 7.4. These results show that these polyelectrolytes interact with BSA and change its conformation. However, from the results of circular dichroism (CD) spectra, the reduced molecular ellipticity at 222 nm ($[\theta]_{222}$), which reflects the α-helix content of BSA, was scarcely changed, although that at 292 nm ($[\theta]_{292}$), which is the chirality of Trp, was changed to a certain extent by such a complexation. Therefore, it was found that BSA may not be denatured by the complexation under these conditions. That is, the change of the fluorescence of Trp may reflect the local conformational change around the Trp residue. Since fluorescence is quenched in many ways [21], λ_{em} is used hereafter as a parameter for

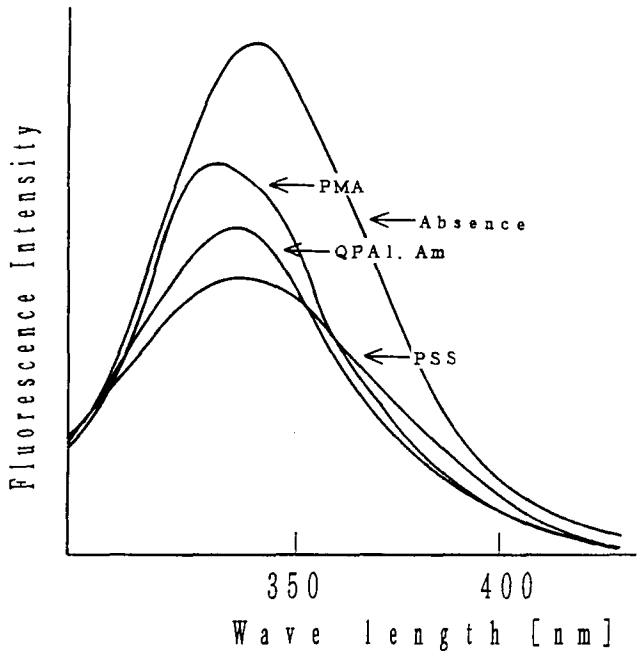

FIG. 2. Fluorescence spectra of BSA in the absence or presence of polyelectrolytes at pH 7.4. [BSA] = 4×10^{-2} (g/L). [Polyelectrolyte] = 2×10^{-4} (unit mol/L).

the conformational change of albumin induced by the interaction with polyelectrolytes.

Figure 3 shows the pH dependence of λ_{em} of the Trp of BSA in the presence of polycations (A) or polyanions (B). After adding polycations, a slight blue shift of λ_{em} was observed only in the pH region higher than the isoelectric point (pI = 4.7) of BSA. The difference of the structural formulas of polycations exerted little influence upon λ_{em}. A curdy precipitate was observed in a more concentrated condition. Thus it is found that polycations can interact randomly with anionic sites of BSA through electrostatic interaction and induce a slight conformational change around the Trp of BSA. On the other hand, polyanions induce a marked blue shift of λ_{em} in pH regions lower than about 9, where the net charge of BSA is negative. It is well known that albumin contains specific binding sites for drugs (Site I, Site II, and Site III; mainly Site I and Site II) [22], where basic amino acids are localized. Acidic compounds (e.g., anionic surfactants, fatty acids, and so on) are bound to these binding sites more easily than are cationic compounds [23]. BSA has two Trp's [17]; one (Trp^{212}) of the two exists in the neighborhood of drug binding Site II [24]. These results suggest that polyanions interact selectively with Site II of BSA even in the alkaline pH region (pH > pI) to change the orientation of Trp^{212}. However, λ_{em} was shifted further toward a shorter wavelength with a decrease of pH. This seemed to be due to a conformational change around another Trp (Trp^{134}) that occurs when the net charge of BSA becomes positive to facilitate the formation of PEC with polyanions. On the other hand, in the PMA system, λ_{em} showed some red shift at very low pH (pH < 5). This seemed to occur when the interaction

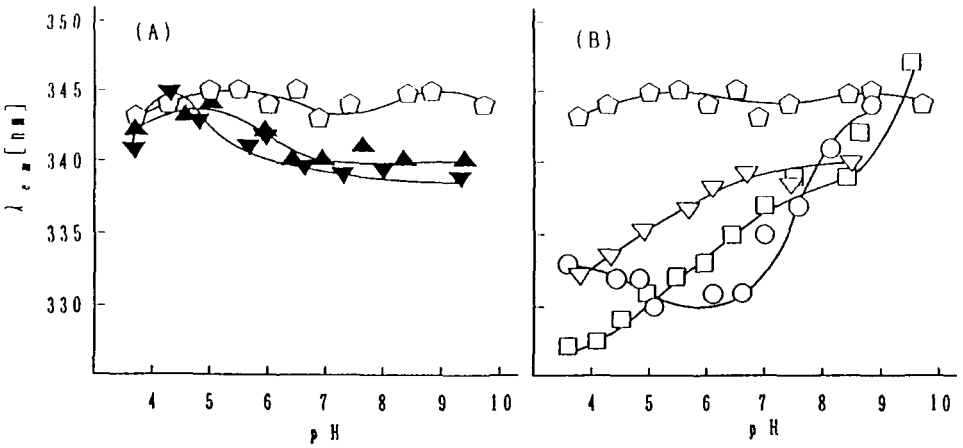

FIG. 3. pH dependence of λ_{em} of tryptophan of BSA in the presence of polyelectrolytes in water. (A) polycations, (B) polyanions. (○) BSA, (▲) BSA + QPAl.Am, (▼) BSA + PQE, (○) BSA + PMA, (□) BSA + PSS, (▽) BSA + PNMA. [BSA] = 4×10^{-2} (g/L). [Polyelectrolyte] = 2×10^{-4} (unit mol/L).

between BSA and PMA was weakened, since self-assembly of PMA took place in such a pH region (pK_a of PMA is about 5.2) [25].

By using HSA, which contains one Trp (Trp^{214}) in the neighborhood of binding Site II, the pH dependence of λ_{em} was not observed in the presence of polyanions (Fig. 4). This result may be due to selective interaction of polyanions with binding Site II of HSA. It was reported that a compound containing both a benzene ring and an anionic site (especially a sulfonic acid group) interacts strongly with Site II [26]. Therefore, PSS probably interacts most strongly with Site II in HSA and is the most effective polyanion for the shift of λ_{em}. The blue shift of λ_{em} induced by PAMPS (which contains no benzene ring but has sulfonate residues) was similar to that of PMA. On the other hand, the effect of QPAl.Am (a polycation) on the conformational change around Trp was observed in pH regions higher than the pI of HSA, which is similar to the BSA results mentioned above.

It is well known that when the emission spectrum of a donor compound and the adsorption spectrum of an acceptor compound overlap and the distance between the two compounds is shorter than the Förster radius, energy transfer takes place. By utilizing such a phenomenon, Kasai et al. [24] calculated the distance from Trp to the binding sites in HSA. In order to discuss the mechanism of the interaction of albumin with polyelectrolytes in more detail, the energy transfer from Trp (a donor) in albumin to anthryl groups (an acceptor, Anth) in polyelectrolytes was evaluated. Figure 5 shows a typical fluorescence spectrum of BSA in the presence of an Anth-loaded polyanion, PMA-A, when Trp was excited at 280 nm. The emission of Anth, as well as of Trp, was also observed, which means that the energy transfer takes place between them and that they are close to each other. The efficiency of the energy transfer $(I^A/I^T)'$ from Trp of albumin to Anth of polyelectrolytes is calculated by the following equation:

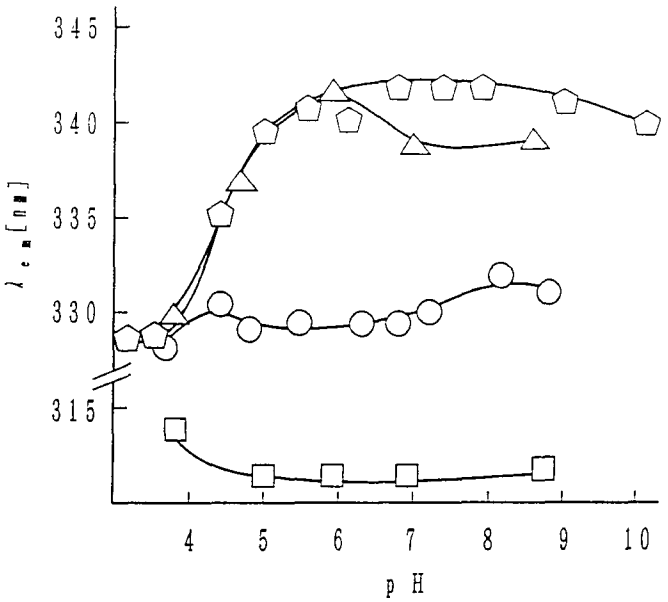

FIG. 4. pH dependence of λ_{em} of tryptophan of HSA in the presence of polyelectrolytes in water. (○) HSA, (△) HSA + QPAl.Am, (○) HSA + PMA, (□) BSA + PSS. [HSA] = 4×10^{-2} (g/L). [Polyelectrolyte] = 1×10^{-4} (unit mol/L).

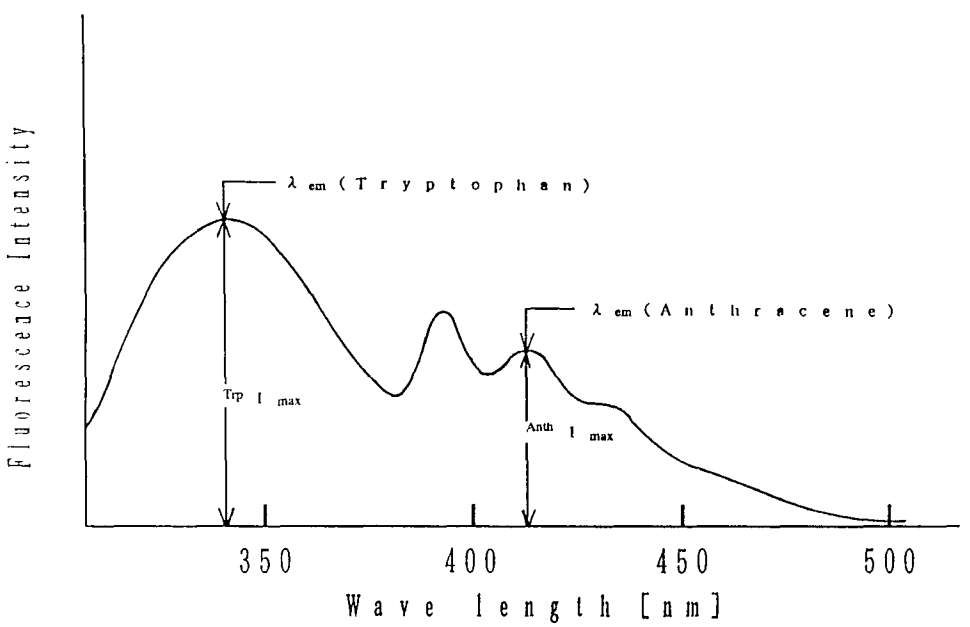

FIG. 5. Typical fluorescence spectrum of BSA with Anth-loaded polyelectrolytes. [BSA] = 4×10^{-2} (g/L). [Polyelectrolyte] = 2×10^{-4} (unit mol/L).

$$(I^A/I^T)' = \frac{^{Anth}I_{max}}{^{Trp}I_{max}} \times \frac{I_{rel}^{Anth}}{I_{rel}^{Trp}} \quad (1)$$

where $^{Anth}I_{max}$ (~420 nm) and $^{Trp}I_{max}$ (~345 nm) are the fluorescence intensities of Anth and Trp respectively, in coexistence with albumin and Anth-loaded polyelectrolytes [excitation wavelength (λ_{ex}) = 280 nm; see Fig. 5]. I_{rel}^{Anth} is the fluorescence intensity of Anth in polyelectrolytes excited at 365 nm in the same system mentioned above, and I_{rel}^{Trp} is that of Trp when albumin interacted with polyelectrolytes without Anth groups (λ_{ex} = 280 nm; see Fig. 2).

Figure 6 shows the pH dependence of $(I^A/I^T)'$ when Anth-loaded polycations (A) or Anth-loaded polyanions (B) were added to the BSA solution. Because the $(I^A/I^T)'$ values are very low in a wide pH region for the polycation–BSA system, polycations interact with anionic sites far from the Trp residue even in a high pH

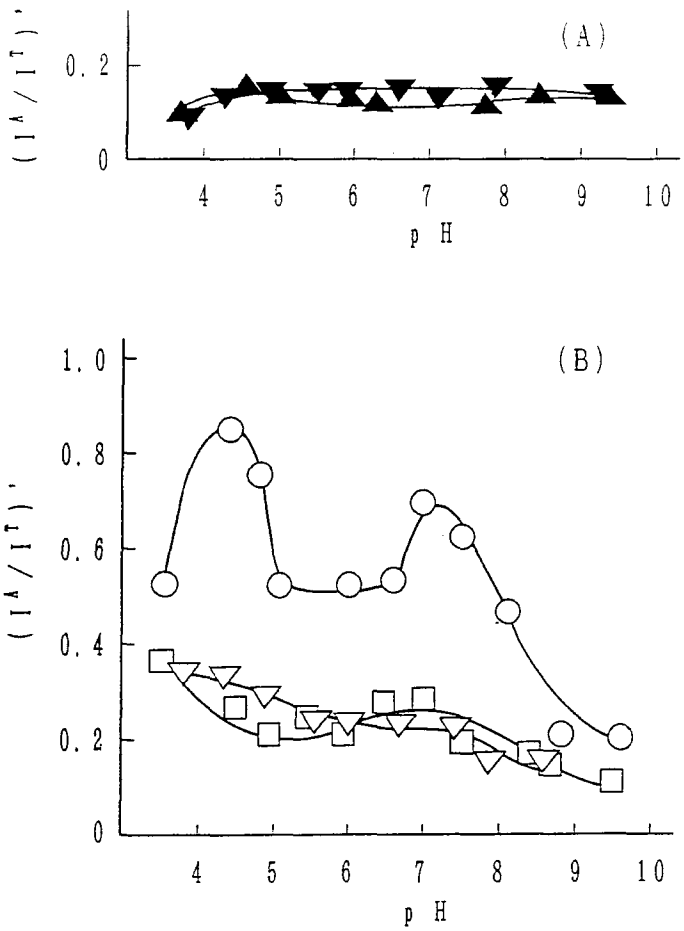

FIG. 6. pH dependence of $(I^A/I^T)'$ by the complexation of BSA with polycations (A) and polyanions (B). (▲) PQP-A, (▼) PQE-A, (○) PMA-A, (□) PSS-A, (▽) PNMA-A. [BSA] = 4×10^{-2} (g/L). [Polyelectrolyte] = 1×10^{-4} (unit mol/L).

region. On the other hand, when Anth-loaded polyanions were added to the BSA solution, $(I^A/I^T)'$ values were much higher than those of Anth-loaded polycations. This result suggests that polyanions interact with the site very close to Trp, probably a drug binding Site II. $(I^A/I^T)'$ increased in the order PSS-A $\simeq$ PNMA-A $<$ PMA-A. Such a difference in $(I^A/I^T)'$ seems to be due mainly to the relative position of the Anth and ionic groups; that is, as shown in Fig. 1, the ionic sites of PSS-A and PNMA-A were at some distance from the main chain, but the ionic sites of PMA-A and Anth of these polyanions were close to the main chain. So, if these polyanions interact with Site II of BSA in the same manner, Anth's of PSS-A and PNMA-A might be separated from Trp by a steric hindrance greater than that of PMA-A. On the whole, $(I^A/I^T)'$ increased with a decrease of pH since the interactions of BSA with these polyanions were strengthened at low pH, as shown in Fig. 3. Moreover, two peaks, at pH $\simeq$ 4.5 and pH $\simeq$ 7.0, were observed, especially in the PMA-A system. A similar tendency [$(I^A/I^T)'$ was increased in the order PQP-A $<$ PSS-A $<$ PMA-A] was also obtained in the HSA system, but no peak was observed, even in the PMA-A system. Therefore, the two peaks observed in the BSA system seemed to be caused by the energy transfer from another Trp^{134}, which exists far from drug binding sites, to Anth groups. It is well known that albumin shows two principal conformational transitions that depend on pH; one is the N-F transition, which takes place in pH region from 3.8 to 4.5 [27], and the other is the N-B transition, from pH 7.0 to 9.0 [28]. In these transition pH periods, Trp was easily moved [29] and the drug binding abilities of the binding sites were increased [30]. From these facts it is believed that PMA-A is the most sensitive to those minor conformational changes of BSA, since Anth and ionic residues are close to each other in PMA-A.

Warfarin (War) and dansyl-L-proline (DNP) have been widely used as fluorescent probes which bind specifically to Site I and Site II, respectively [26]. When these probes are bound to binding sites, energy transfers from Trp to War or DNP take place; that is, the fluorescence of Trp disappears at the same time that the emission of War (360–390 nm) or DNP (460–530 nm) appears. Figure 7 shows the fluorescent spectra of the BSA–War complex in the presence or absence of polyelectrolytes. In the presence of a polycation (QPAl.Am), the fluorescence intensity of War became slightly higher and its λ_{em} was shifted to a longer wavelength, but the emission of Trp was not observed. Since the fluorescence spectrum of War showed similar changes with an increase of solution pH, QPAl.Am was considered to behave as a polybase which made the microenvironment around War more basic. And War, which was bound to Site I, was not substituted for by the polycation. However, on adding polyanions (PMA and PSS), the reduction of fluorescence intensity and a blue shift of λ_{em} of War were observed, and at the same time the fluorescence of Trp was restored to a certain extent. These results suggest that part of War might be substituted for polyanions and that the microenvironment around the bound War might be acidic. Similar results were obtained in the HSA system as well as in the BSA system. That is, it was found that these polyanions interact weakly with Site I and polycations do not interact directly with Site I.

Figure 8 shows the change of fluorescence spectra of the BSA–DNP complex upon the addition of polyelectrolytes. A polycation had no effect on the spectrum of DNP. This result suggests that a polycation interacts with sites far from Site II. In the presence of polyanions, the emission of DNP was extremely reduced and that of Trp was restored. Especially in the PSS system, only a very weak fluorescence of

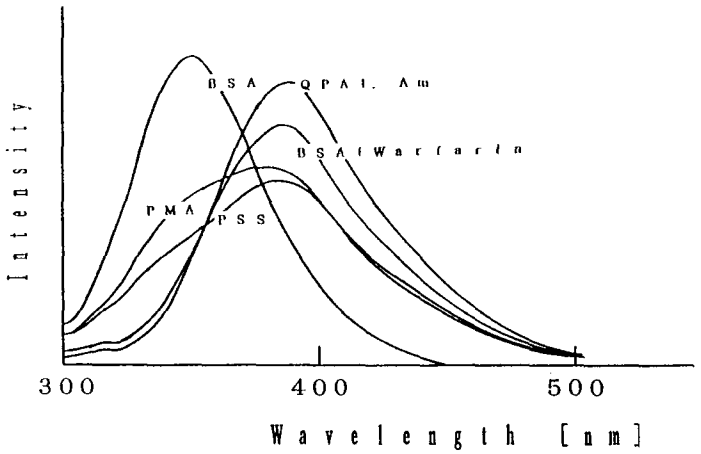

FIG. 7. Fluorescence spectra of warfarin-bound BSA in the presence of polyelectrolytes at pH 7.4. [BSA] = 4×10^{-2} (g/L). [Warfarin] = 3×10^{-5} (mol/L). [Polyelectrolyte] = 1×10^{-4} (unit mol/L).

DNP with λ_{em} at 525 nm, which was the same λ_{em} as DNP in aqueous solution, was observed. In the case of HSA as well as of BSA, similar results were obtained. That is, almost all the DNP seemed to be substituted for polyanions. From these results it was found that the interacting sites in BSA with these polyanions were Site II, and that the interaction force of PSS with Site II was much stronger than that of PMA. This fact is consistent with the results of Figs. 3 and 4.

The interaction mechanisms between BSA and polyelectrolytes are summarized schematically in Fig. 9.

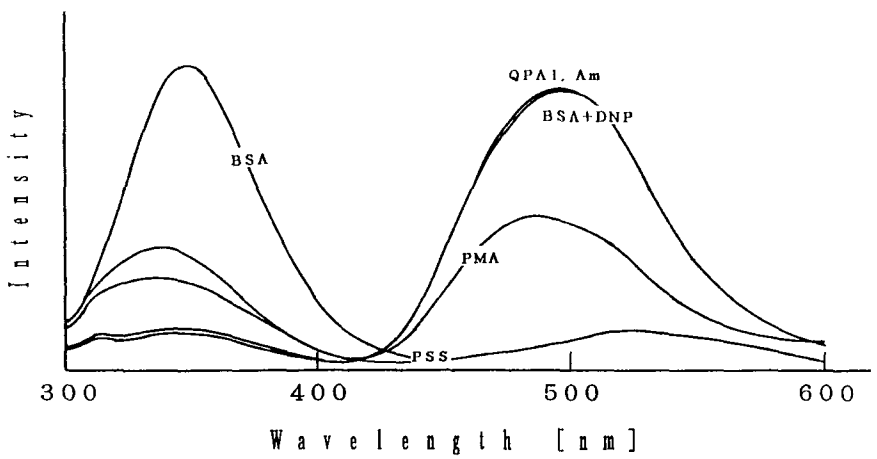

FIG. 8. Fluorescence spectra of dansyl-L-proline (DNP)-bound BSA in the presence of polyelectrolytes at pH 7.4. [BSA] = 4×10^{-2} (g/L). [DNP] = 3×10^{-5} (mol/L). [Polyelectrolyte] = 1×10^{-4} (unit mol/L).

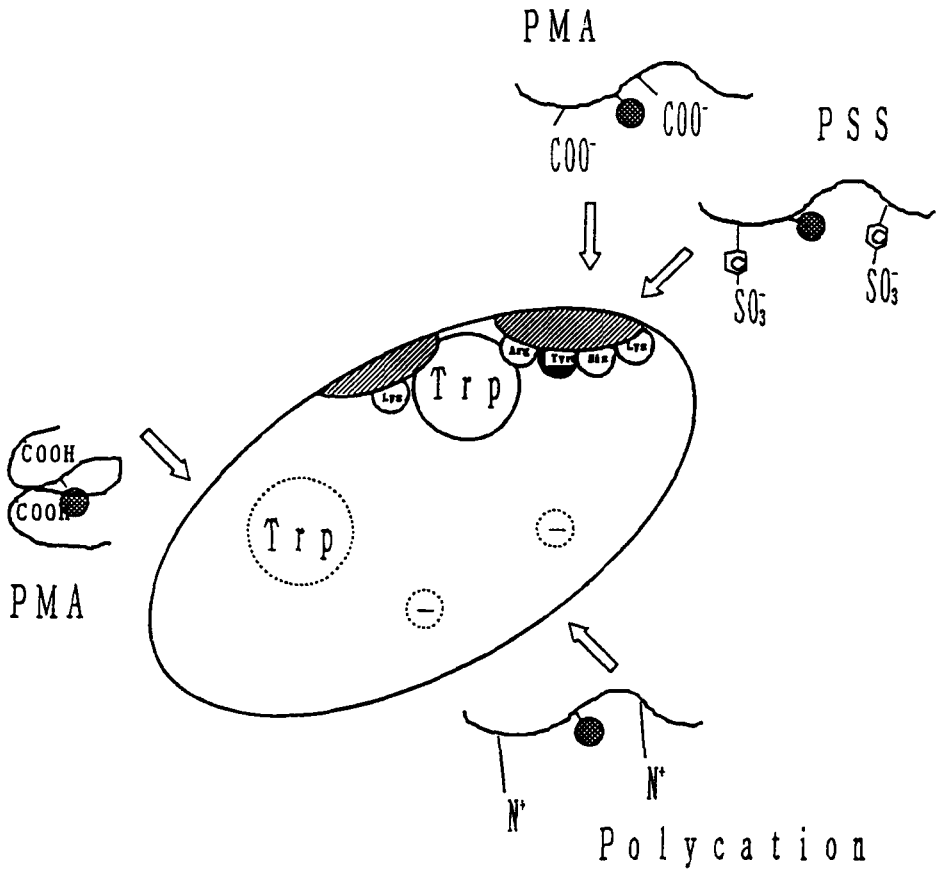

FIG. 9. Schematic representation of the complexation of albumin with polyelectrolytes.

1. Polycations can interact with albumin only in the pH region higher than an isoelectric point (pI = 4.3), mainly through electrostatic interaction at a position far from the drug binding sites. The conformational change around Trp of albumin was small.

2. Polyanions can interact with albumin over a wide pH range. In neutral to alkaline pH regions, polyanions may be bound with specificity to the drug binding sites of albumin, especially binding Site II. The interaction force of PSS with Site II is stronger than that of PMA, because PSS has both a sulfonic acid residue as an ionic site and a benzene ring.

These facts are very important in any consideration of the interaction mechanism of proteins with artificial macromolecules, not only in vitro but also in vivo.

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