[Chem. Pharm. Bull. 32(6)2395—2400(1984)]

Study of Protein Adsorption on Glass Surfaces with a Hydrophobic Fluorescent Probe

TAKAHARU MIZUTANI* and AYAKO ASAOKA

Faculty of Pharmaceutical Sciences, Nagoya City University, Tanabe-dori, Mizuho-ku, Nagoya 467 Japan

(Received September 26, 1983)

The relationship between the affinity of proteins for glass surfaces in a water medium and the fluorescence intensity of a probe [7-(*p*-methoxybenzylamino)-4-nitrobenz-2-oxa-1,3-diazole (MBD)] of hydrophobic areas on proteins was studied. Proteins displaying a weak affinity for glass surfaces, such as bovine serum albumin and horse-radish peroxidase, showed stronger fluorescence than those displaying a stronger affinity for glass surfaces, such as serum globulin and fibrinogen. Compatible results were obtained in the chromatography of protein mixtures, such as serum or extract of liver, on porous glass columns. These results suggest that hydrophobic interactions do not participate in the adsorption of proteins on glass surfaces.

Keywords——protein adsorption; porous glass; hydrophobic probe; fibrinogen; albumin; fluorescent probe; glass surface

We have been studying protein adsorption on glass surfaces in order to evaluate the storage characteristics of pharmaceutical protein preparations such as vaccines or hormones in glass containers.[1-3] We have also developed an adsorption chromatography method for proteins on porous glass.[4-6] The adsorption of proteins on glass surfaces is caused by several factors. One of the major factors is the ionic bonding between anionic silanol on glass surfaces and cationic amine residues on proteins. However, it was found that proteins show the strongest affinity for glass surfaces at their isoelectric points.[7] Therefore, other factors, such as hydrogen bonding, hydrophobic interactions and/or van der Waals forces (molecular forces), might participate in the adsortion of proteins on glass surfaces. Hydrogen bonding had no effect on adsorption because the proteins were well adsorbed in 8 M urea or 6 M guanidine hydrochloride, which are generally considered to destroy hydrogen bonds.[7]

The participation of hydrophobic interactions in the adsorption of proteins was suggested by the findings that interferons adsorbed on porous glass were desorbed with hydrophobic solutes, such as $(alkyl)_4$-ammonium chloride[8] or ethyleneglycol.[9] We compared the contents of aliphatic amino acids in proteins having strong affinity and those having weak affinity, but we could not obtain clear evidence for the participation of hydrophobic interactions.[10] The purpose of this investigation was to clarify the participation of hydrophobic interactions in protein adsorption by using a hydrophobic fluorescent probe [7-(*p*-methoxybenzylamino)-4-nitrobenz-2-oxa-1,3-diazole (MBD)].

Experimental

The porous glass used was CPG-10 240 Å (Electro-Nucleonics, N.J., U.S.A.) which was composed of 96% silica glass and had a surface area of 97 m^2/g. The glass was packed in a column (0.6 cm internal diameter × 9 cm, length). The column was washed with 0.1% sodium dodecyl sulfate (SDS), distilled water and a chromic acid mixture and then thoroughly rinsed with distilled water. Protein solutions were applied to the column for the adsorption experiments. Standard proteins used were bovine serum alubmin (BSA, Armour Pharm. Co., Chicago, Ill., U.S.A.), rabbit serum globulin (prepared in this laboratory), bovine fibrinogen (Katayama Chem., Osaka, Japan), horse-radish peroxidase

(HRP, Sigma Chem., St. Louis, Mo., U.S.A.), bovine hemoglobin (Sigma Chem.), and chicken egg lysozyme (Sigma Chem.). These proteins were not further purified and were dissolved in distilled water at a concentration of 1 mg/ml (pH 7.6—8.4) and then 2 ml was loaded on the column. After adsorption on the column for 10 min, 1 h, or 16 h at room temperature (20—25 °C), proteins were eluted with 0.2 M glycine buffer at pH 8.0. Natural protein mixtures subjected to the adsorption chromatography were rabbit serum and a murine liver extract (the 8000 × *g* supernatant). Adsorption chromatography of these mixture was carried out on the CPG column according to the previous report.[11] Protein concentration in the eluate from the column after adsorption was determined by measuring the absorbance at 280 nm, using the following $E^{1\%}_{1cm}$ values: 6.7 for BSA, 11 for HRP, 14 for globulin and 15 for fibrinogen. The concentrations of standard proteins in the eluate from the columns were adjusted to 0.2 mg/ml for BSA and HRP, and to 0.5 mg/ml for globulin and fibrinogen with 0.2 M glycine buffer for fluorescence measurements. Protein concentration in the eluate obtained by adsorption chromatography of the protein mixtures was determined by the method of Lowry *et al.*[12] and was adjusted to 0.2—0.5 mg/ml.

Protein solutions (2 ml) were mixed with 2 ml of 10 μM MBD (Dojindo Lab., Kumamoto, Japan), a fluorescent probe of hydrophobic areas in proteins, in 0.2 M glycine buffer.[13] Fluorescence was measured at 25 °C with a Shimadzu RF510 spectrofluorophotometer at an excitation wavelength of 335 nm. The fluorescence emission spectrum of each protein–MBD mixture was corrected by subtraction of the spectrum of 5 μM MBD solution alone.

Results and Discussion

The order of elution of proteins from the porous glass column was HRP, BSA, hemoglobin, globulin, fibrinogen and lysozyme.[10] These results reflect the order of affinity for glass surfaces (HRP has the weakest affinity among these proteins). The difference of affinity between pairs of proteins was studied by the elution of a mixture on a CPG column. The results are shown in Fig. 1. The upper part in Fig. 1 shows the elution pattern of a mixture of BSA and HRP at a concentration of 0.5 mg/ml each in phosphate-buffered saline at pH 7.2. In this experiment, the concentration of HRP was determined by the measurement of absorbance at 403 nm. The concentration of total protein (BSA and HRP) was obtained from the absorbance at 280 nm. The ratio of A_{403} to A_{280} of the solutions in tubes 6—10 was about 0.8. This value is higher than that of the original solution (ratio 0.3). Therefore, the eluate in tubes 6—10 contained more HRP than did the original solution. As the elution increased, the ratio of A_{403} to A_{280} (the content of HRP) approached the value of the original solution. These results show that BSA has a stronger affinity for glass surfaces than HRP. The lower part of Fig. 1 shows the elution pattern of a mixture of HRP and fibrinogen on a CPG column. The ratio of A_{403} to A_{280} showed that HRP appeared in the initial eluate (tubes 7—10) but fibrinogen was not eluted. These results are similar to the pattern in the upper part of Fig. 1. Fibrinogen had a stronger affinity for glass surfaces than HRP. The elution pattern of a mixture of hemoglobin and BSA (data not presented) showed that both proteins were well adsorbed. Neither protein was found in the initial eluate from the CPG column. After fifty column-volumes of protein solution had been loaded on the column, a small amount of BSA was eluted but hemoglobin was adsorbed on the column as a band of brown color at the top of the column. This result showed that hemoglobin has a stronger affinity for glass surfaces than BSA. From these results with three mixtures and the chromatographic pattern of serum,[5] the order of the affinity of proteins (weak to strong) was concluded to be HRP—BSA—hemoglobin—globulin—fibrinogen. These proteins were elutable from the column. Lysozyme and chymotrypsinogen A have strong affinity and are difficult to elute from the column.[11]

If protein adsorption on glass surfaces involves hydrophobic interactions, the proteins with strong surface affinity would bind larger amounts of the hydrophobic probe MBD and the proteins with weak affinity would bind smaller amounts of the probe. Therefore, MBD binding to proteins was examined.

Proteins were best adsorbed on glass surfaces from distilled water among many kinds of solvent tested. The proteins described above were all desorbed with glycine buffer at pH 8.0.

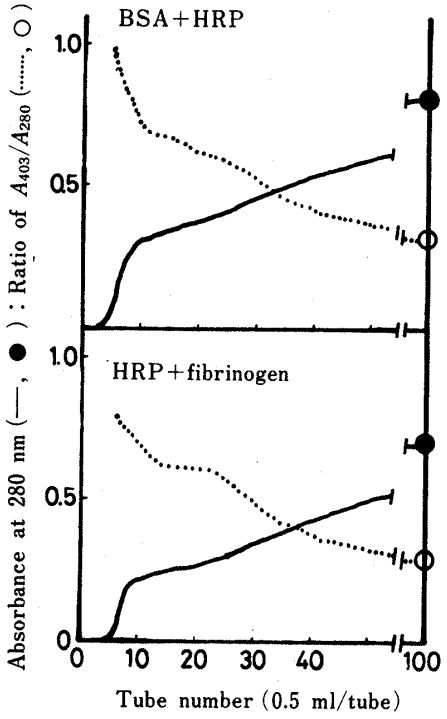

Fig. 1. Adsorption Patterns of Mixtures on Porous Glass Columns

Upper, a mixture of bovine serum albumin and horse-radish peroxidase (HRP) (each 0.5 mg/ml); lower, a mixture of HRP and bovine fibrinogen (each 0.5 mg/ml). The column size was 0.6 cm × 9 cm. The solid lines and the closed circles show absorbance at 280 nm (total proteins); the dotted lines and the open circles show the ratio of A_{403} (HRP) to A_{280}.

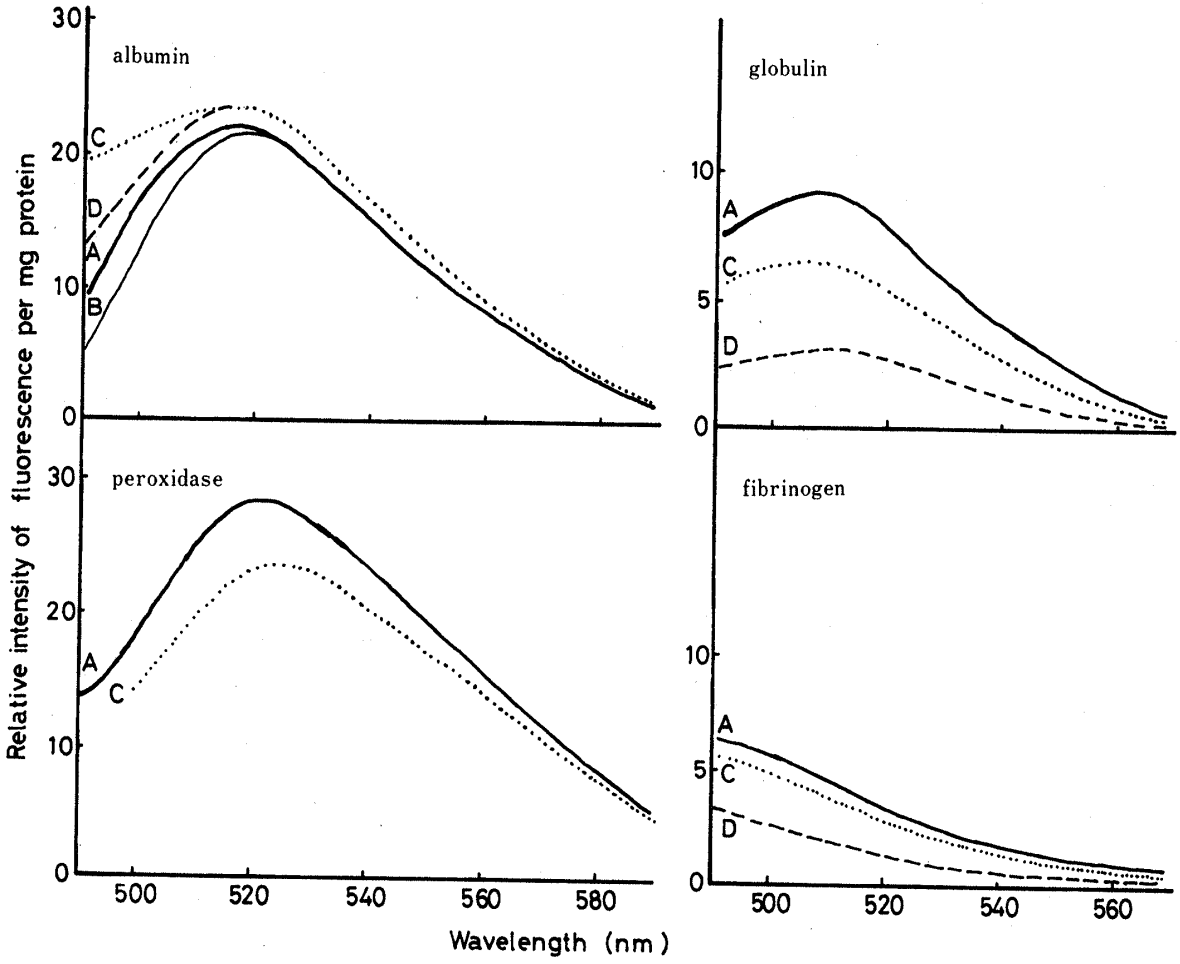

Fig. 2. Fluorescence Spectra of Protein-Fluorophore Complexes

Thick solid lines (A), non-treated (control); thin solid line (B), adsorption for 10 min on a CPG column; dotted lines (C), adsorption for 1 h; broken lines (D), adsorbed for 16 h. Experimental conditions are described in the text.

Therefore, BSA was dissolved in distilled water and applied to the CPG column. The eluates from the column with the glycine buffer were mixed with MBD solution and the fluorescence emission spectra were measured, as described in Experimental. The spectra of the relative fluorescence intensity per protein concentration (mg/ml) for BSA are shown in the upper left part of Fig. 2. The spectrum of BSA adsorbed for 10 min was identical with that of non-adsorbed BSA (control). This result shows that the adsorption of BSA on glass surfaces for a short time does not influence the amount of MBD bound on BSA. The spectrum of BSA adsorbed for 1 h showed a slight intensity increase. This result suggests that hydrophobic areas binding MBD were somewhat increased by adsorption and that the ordered structure of BSA changed during adsorption for 1 h. The fluorescence spectrum of BSA adsorbed for 16 h was similar to that of BSA adsorbed for 1 h. Thus, in the case of BSA, the intensity of the fluorescence (the amount of MBD bound on BSA) hardly changed during adsorption and was almost independent of the adsorption time.

The lower left part of Fig. 2 shows the spectra of HRP. The spectrum of HRP adsorbed for 16 h was almost identical to that of HRP adsorbed for 1 h. The fluorescence spectra of HRP were influenced by the optical behavior of HRP itself in the wavelength range of 480—540 nm. Therefore, the HRP data are less accurate than those for the other proteins. The fluorescence intensity of HRP adsorbed for 1 h decreased to 85% of that of untreated HRP. This change is considered to be due to the denaturation of HRP.

The upper right part of Fig. 2 shows the fluorescence spectra of rabbit globulin. The intensity of the globulin fluorescence was about one-tenth of the intensity of BSA and HRP. This result showed that the globulin hydrophobic area, which binds MBD, is less than that of BSA or HRP. The fluorescence intensity decreased as the time of adsorption on CPG increased. This is similar to the HRP results, and suggests that the globulin is denatured during adsorption. The major secondary strucrure in globulin is β-structure,[14] so a change in the content of β-structure might be related to the decrease of fluorescence intensity with increasing adsorption time.

The lower right part of Fig. 2 shows the fluorescent spectra of fibrinogen. The intensity of fibrinogen was lower than that of globulin and decreased with increasing time of adsorption as was the case with globulin. The basic protein lysozyme had a strong affinity for glass surfaces and as a result, desorption of lysozyme from CPG was difficult.[11] Lysozyme is adsorbed on glass surfaces by ionic bonding. A mixture of lysozyme and MBD did not show any fluorescence, indicating that lysozyme has little if any hydrophobic area able to bind MBD on its surface. The measurement of the fluorescence of hemoglobin was not done because the fluorescence spectrum was interfered with at 480—600 nm by the absorption of heme groups in hemoglobin.

TABLE I. The Influence of Adsorption Time on the Relative Fluorescence Intensity at 530 nm[a]

Protein	Non-adsorption	Time of adsorption		
		10 min	1 h	16 h
Horse-radish peroxidase	27	25	23	23
Bovine serum albumin	19	19	21	20
Globulin	5.7	—	3.8	2.0
Fibrinogen	2.6	3.1	2.1	1.1
Lysozyme	0	—[b]	—[b]	—[b]

a) The values are normalized with respect to the protein concentrations.
b) These values were not determined because lysozyme was not desorbed.

The relative fluorescence intensities at 530 nm per protein concentration are summarized in Table I, which also shows the changes in intensity with increasing adsorption time on the glass. Generally, the intensity of MBD bound to proteins decreased with increasing time of adsorption on CPG, with the exception of BSA, as shown in Table I. These results indicate that hydrophobic areas on proteins decreased with increasing time of adsorption and also suggest that the proteins were denatured on the glass surfaces. Changes of the secondary structure of proteins[15] and conformational rearrangements inside protein molecules[16] during adsorption have already been reported and we showed that proteins adsorbed on glass were inactivated during storage.[17]

The order of the fluorescence intensity per protein concentration in Table I varies inversely with the affinity of the protein for the glass surfaces as described above (Fig. 1). HRP having the weakest affinity among these proteins bound the most MBD. Lysozyme having the strongest affinity did not bind any MBD (Table I). Thus, proteins having more hydrophobic area had weaker affinity for glass surfaces and were more readily desorbed from the CPG column. Proteins which did not bind MBD had a strong affinity for glass surfaces and were hardly eluted from the CPG column. Thus, it can be concluded that protein adsorption on glass surfaces is not related to hydrophobic interactions.

This conclusion is based on the use of standard proteins, and so we also investigated protein mixtures such as rabbit serum and an extract of murine liver. Figure 3 shows the chromatographic patterns of these protein mixtures, as well as the results of fluorescence

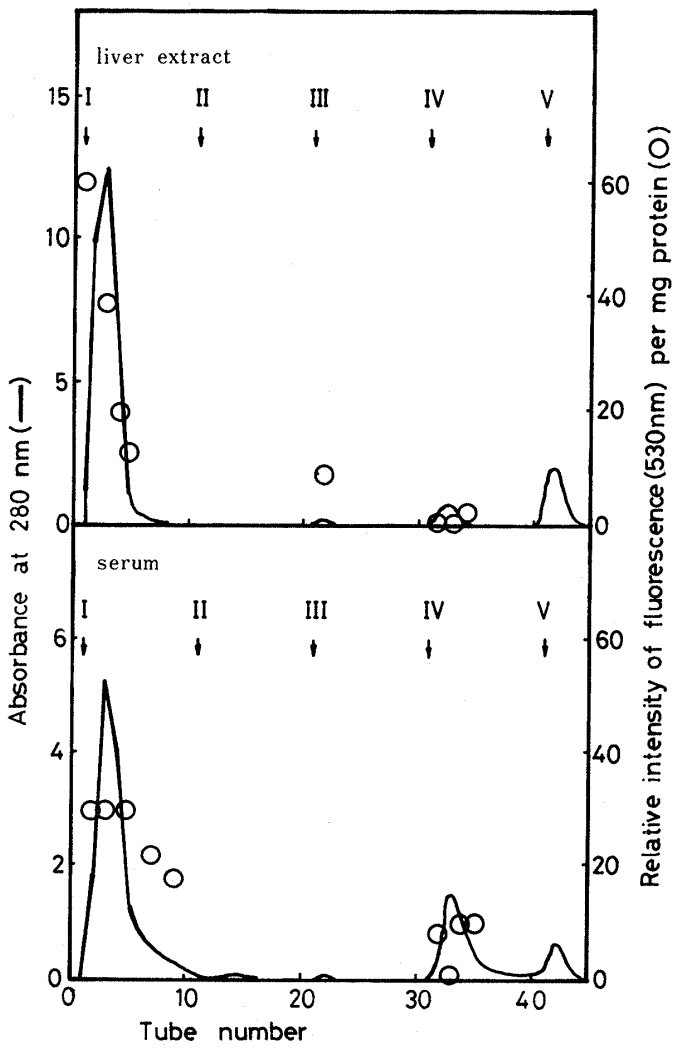

Fig. 3. Chromatographic Patterns of Protein Mixtures on Porous Glass Columns and the Fluorescence Intensity of Eluates

Upper, an extract of murine liver; lower, rabbit serum; solid lines, absorbance at 280 nm; open circles, the relative intensity of fluorescence per protein concentration. Buffers used for elution are phosphate-buffered saline at pH 7.2 for I, 0.2 M Tris-HCl at pH 7.6 for II, 0.2 M glycine at pH 8.0 for III, 0.2 M glycine at pH 8.7 for IV and 0.1% SDS for V. Fraction volume was 1.2 ml.

intensity measurements at 530 nm. In both cases, proteins with weak affinity for CPG, *i.e.*, those eluted first from the column, showed a stronger intensity of fluorescence than proteins that eluted later, *i.e.*, those with stronger affinity for the glass surfaces. These results are consistent with the results for standard proteins as shown in Fig. 2. Thus, proteins with a weak affinity for glass surfaces bound more hydrophobic probe MBD and proteins with a strong affinity for glass surfaces bound less MBD. Thus, the present results exclude the possibility of the participation of hydrophobic interactions in the adsorption of proteins on glass surfaces. Hydrogen bonding is also not related to the adsorption.[7] It is possible for van der Waals molecular forces to participate in protein adsorption,[18] but ionic bonding must be a major factor.

References

1) T. Mizutani, *J. Pharm. Sci.*, **69**, 1226 (1980).
2) T. Mizutani, *J. Pharm. Sci.*, **70**, 493 (1981).
3) T. Mizutani, *J. Pharm. Pharmacol.*, **34**, 608 (1982).
4) T. Mizutani and A. Mizutani, *J. Chromatogr.*, **111**, 214 (1975).
5) T. Mizutani and A. Mizutani, *J. Chromatogr.*, **120**, 206 (1976).
6) T. Mizutani and A. Mizutani, *Anal. Biochem.*, **83**, 216 (1977).
7) T. Mizutani and A. Mizutani, *J. Pharm. Sci.*, **67**, 1102 (1978).
8) K. C. Chadha and E. Sulkowski, *Prep. Biochem.*, **11**, 467 (1981).
9) I. A. Braude, V. G. Edy and E. DeClercq, *Biochim. Biophys. Acta*, **580**, 15 (1979).
10) T. Mizutani, *J. Colloid Interface Sci.*, **79**, 284 (1981).
11) T. Mizutani and A. Mizutani, *J. Chromatogr.*, **168**, 143 (1979).
12) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
13) R. A. Kenner and A. A. Aboderin, *Biochemistry*, **10**, 4433 (1971).
14) R. Huber, J. Deisenhofer, P. M. Colman, M. Matsushima and W. Palm, *Nature* (London), **264**, 415 (1976).
15) B. M. C. Chan and J. L. Brash, *J. Colloid Interface Sci.*, **84**, 263 (1981).
16) W. Norde and J. Lyklema, *J. Colloid Interface Sci.*, **71**, 350 (1979).
17) T. Mizutani, *J. Pharm. Sci.*, **69**, 279 (1980).
18) B. Chu, "Molecular Forces: Based on the Baker Lectures of P. J. M. Debye," John Wiley and Sons, Inc., New York, 1967.