HUMAN SERUM ALBUMIN. A STUDY OF THE NATURE OF ITS HYDROPHOBIC BINDING SITES

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Spectroscopic labels and hydrophobic chromatography on two different supports were used to compare the size and accessibility of the hydrophobic binding sites of human serum albumin with the accessibility of non-polar residues on the surface of other globular proteins. The binding of the labels 1-alkyl-4-(3-ethoxy-4-hydroxystyryl)pyridinium bromides (HPB) with alkyl chains of different length was investigated in the ultracentrifuge and by spectrophotometry. n-Butyl (C_4-HPB) and decyl (C_{10}-HPB) labels bind to albumin with association constants of $8 . 10^3$ and $4 . 10^4$, respectively, at pH 5·50, and with constant of $2{\cdot}6 . 10^5$ for the C_{10}-HPB label at pH 9·2. Whereas C_4-HPB interacts with the site of local polarity of albumin not different from the bulk solution, both the C_{10}-HPB and C_{16}-HPB on the other hand bind to hydrophobic sites, where the solvation of the chromophore is largly reduced as evidenced by the 46 nm shift to higher wavelengths in its spectrum. For other proteins the shift was less then 5 nm. Ten molecules of C_{10}-HPB and four molecules of C_{16}-HPB can be attached to one molecule of albumin. The changes in the spectrum of the bound label induced by palmitate reveal that these binding sites are essentially the same as those for fatty acids. From chromatographic experiments with labeled albumin at different pH carried out on Octyl-Sepharose and Spheron the conclusion was made that the latter support interacts preferentially with the non-polar side chains on the surface of proteins. The retention and the recovery of albumin and defatted albumin was investigated as a function of salt and alcohol concentration and compared with the same parameters of other proteins. In agreement with the proposed structure of the domains of albumin[10] evidence was obtained that the outer surface of the albumin molecule is at neutral pH predominantly hydrophilic, and that the exceptionally large hydrophobic areas are localized solely in crevices.

A great deal of work has been devoted to the elucidation of the exceptional properties of albumin and a large bulk of data on its physico-chemical properties have been accumulated[1,2]. Except for the overall shape of the molecule information on the details of its molecular architecture is scarce. This problem was very difficult to approach until the primary structure of albumin[3] had been determined. On the basis of the internal homology in the primary structure of albumin, the results of hydrodynamic and other physical measurements[4–6], and limited proteolysis[5,7–9]

a domain model of the albumin molecule has been proposed[10]. This model shows many interesting features and although not verified it could serve as a base for studies on the structure and function of albumin. We focussed our attention on the size and accessibility of the surface unpolar regions of albumin because certain aspects of the structure of these domains can be verified by this approach.

Evidence has been obtained that the hydrophobic residues undoubtedly play an important role also in the binding of biologically active ligands to albumin, such as fatty acids, bilirubin, heme, tryptophan, some drugs, *etc.*[1,11-15]. Several attempts have been made to relate the hydrophobicity of proteins to some measurable quantity, like partition coefficient[16], shifts or quenching of fluorescence spectra[17,81].

In this paper the hydrophobic chromatography and the spectral label technique originally employed for studies on the solvation of synthetic polymers[19,20] were adopted for the study of hydrophobic binding sites of albumin.

EXPERIMENTAL

Proteins

Human serum albumin (min. 98%) was purchased from Sevac, Czechoslovakia; the dimer content (6%) was determined by polyacrylamide gel electrophoresis. The fatty acids content was 2·1 mol/mol albumin as determined by GLC. Albumin was defatted by twice repeated charcoal treatment at pH 3 (ref.[21]) to 0·1 mol of fatty acids *per* mol of albumin. Ovalbumin (Albumin Egg Art. No. A 5503, 99% cryst., grade V) was from Sigma, St. Louis, Mo. U.S.A. Human gamma globulin was from Sevac, Czechoslovakia, ribonuclease A from Miasokomb., Leningrad, U.S.S.R., egg white lysozyme (LYSF 353881), trypsinogen (Art. No. 3648), and chymotrypsinogen A (Art. No. 5630) were from Worthington Biochem. Corp., Freehold, N.J., U.S.A. Human apohemopexin was prepared according to ref.[22], trypsin isoinhibitors from bull seminal plasma according to ref.[23]. Basic pancreatic trypsin inhibitor (5200 IU/mg) was from Léčiva, Czechoslovakia.

Chromatographic Supports and Substances

Octyl-Sepharose with a higher content of octyl substituents than in the comercial product was prepared from Sepharose CL-4B (Pharmacia Uppsala, Sweden) as described in ref.[24]. Spheron P300 (20—40 μm) which is the macroporous ethylen glycol methacrylate resin was supplied by Lachema, Czechoslovakia. Both supports were washed with alcohol and acetone and traces of ionic groups were removed by diazomethane. The other chemicals used were of analytical or spectral purity grade and supplied by Lachema, Czechoslovakia.

Synthesis of the Labels

The synthesis of 1-(δ-bromobutyl)-4-(3-ethoxy-4-hydroxystyryl)pyridinium bromide (Br-HPB) was described in the previous paper[20]. 1-Methyl-4-(3-ethoxy-4-hydroxystyryl)pyridinium bromide (C_1-HPB) and the corresponding 1-butyl (C_4-HPB), 1-decyl (C_{10}-HPB), and 1-hexadecyl (C_{16}-HPB) derivatives were prepared in an analogous manner.

Determination of the Association Constant for Binding of C_4-HPB and C_{10}-HPB Labels to Albumin

In order to avoid the difficulties caused by the strong tendency of amphiphilic compounds to adsorb to membranes during the dialysis experiments, the ultracentrifuge technique was used. A $3 . 10^{-4}$ mol l^{-1} solution of defatted albumin in 0·1 mol l^{-1} phosphate buffer at pH 5·50 containing different concentrations of labels was centrifuged after a 2 h incubation at 20°C for 7 hours at 58.000 rpm and 20°C in Beckman L5 65 centrifuge using the SW 60 rotor. The upper solution was carefully withdrawn and the concentration of the free labels determined by measurement of the absorbance at 389 nm that was made on a Zeiss PMQ II Spectrophotometer. Spectra were recorded on a Unicam SPP 8000 spectrophotometer.

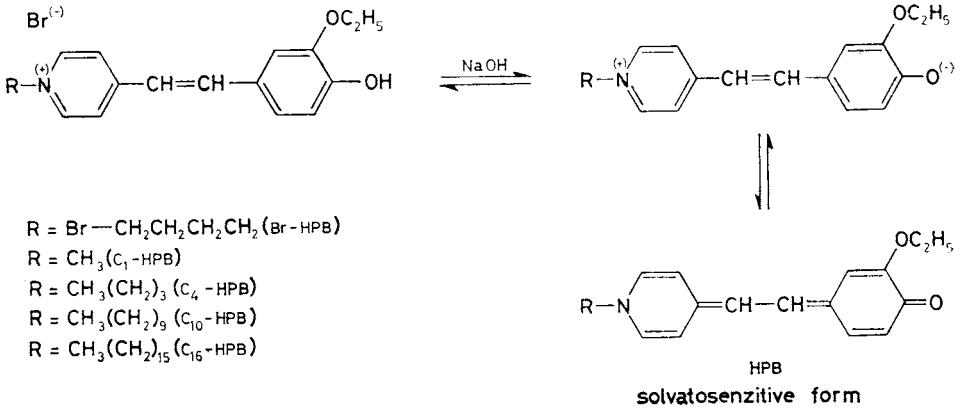
The binding of C_{10}-HPB and C_{16}-HPB to albumin was also followed at pH 9·2 in 0·15 mol l^{-1} borate buffer by spectrophotometric titration monitored at 575 nm.

Reaction of Albumin with 1-(δ-Bromobutyl)-4-(3-ethoxy-4-hydroxystyryl)pyridinium Bromide (Br-HPB)

The modification reaction of the nucleophilic groups of albumin was performed at 2°C in 0·1 mol . l^{-1} acetate, phosphate or borate buffer of the desired pH. Four ml of a $4 . 10^{-4}$ mol l^{-1} solution of albumin and the same volume of a $2·5 . 10^{-2}$ mol l^{-1} solution of Br-HPB were mixed. After 48 hours the reaction mixture was brought to pH 5 and albumin separated from Br-HPB on CM-Sephadex C 25. Albumin was eluted in the void volume by 0·1 mol l^{-1} acetate buffer pH 5·0, but Br-HPB was strongly retained.

Determination of the Local Polarity

The determination of the local polarity on the surface of proteins is based on the spectral shifts of the solvate sensitive charge-transfer band in the visible spectrum of the label, *i.e.* 1-alkyl-4-(3-ethoxystyryl)pridinium bromide (HPB), to form HPB after conversion at an alcaline pH (Scheme 1). The method originally proposed by Kosower[25] and adopted for the study of the influence of the nonpolar polymer backbone of a synthetic polymer on its solvation[19,20] was used in this study for the characterization of the local polarity of hydrophobic binding sites of albumin.



R = Br—$CH_2CH_2CH_2CH_2$ (Br-HPB)
R = CH_3 (C_1-HPB)
R = $CH_3(CH_2)_3$ (C_4-HPB)
R = $CH_3(CH_2)_9$ (C_{10}-HPB)
R = $CH_3(CH_2)_{15}$ (C_{16}-HPB)

SCHEME 1

Chromatographic Experiments

The chromatographic experiments were made on 136 × 7·8 mm precision bore glass columns of Octyl-Sepharose at a flow rate of 20 ml h^{-1} cm^{-2} and on a 246 × 6·24 mm column of Spheron P 300 at a flow rate of 100 ml h^{-1} cm^{-2} at 20°C. The proteins (0·05—0·005 mg) were applied and the chromatography was monitored at 220 or 280 nm by UVM-4 type spectromonitor (proteins) and at 389 nm (for albumin labeled with C_{10}-HPB or C_{16}-HPB).

Labeled albumin used in chromatographic experiments was prepared by mixing the solution of the label with the solution of defatted albumin. After 1 hour at 20°C incubation the protein was separated on a Sephadex G-25 column.

RESULTS

Binding of the Alkyl-Substituted Label (HPB) *to Albumin*

Albumin was labeled by binding of the butyl (C_4-HPB), decyl (C_{10}-HPB), and hexadecyl (C_{16}-HPB) label to the hydrophobic sites of albumin, or by a chemical modification of the nucleophilic surface groups by the 1-(δ-bromobutyl) (Br-HPB) label.

The solubility of C_4-HPB and of C_{10}-HPB enabled us to determine the binding constant by direct equilibrium measurements in aqueous buffers.

It was found that one molecule of C_4-HPB binds to albumin with an association constant $8 . 10^3$. Direct spectrophotometric titration showed that ten molecules of C_{10}-HPB bind to albumin with the association constant $2{\cdot}6 . 10^5$ at pH 9·18. A lower value ($4 . 10^4$) was found at pH 5·5 by equilibrium centrifugation.

The solubility of C_{16}-HPB in aqueous buffers is too low for direct spectrophotometric measurement and therefore the association constant of C_{16}-HPB was not calculated. Four molecules of C_{16}-HPB bind to one molecule of albumin.

Binding Site of Albumin

The spectra of the label substituted by methyl, butyl, decyl, and hexadecyl groups in the presence of a 25 molar excess of albumin are shown in Fig. 1. The label substituted by short alkyl chains (methyl and butyl), free in solution and in the presence of a large (up to 52-fold) excess of albumin, have very similar spectra. No difference was found for C_1-HPB and a small shift only was observed for C_4-HPB upon binding (Table I, Fig. 1).

Significant large shifts of maxima of the charge-transfer band were found for the labels with longer hydrocarbon chains (Table I, Fig. 1). The spectra of C_{10}-HPB and C_{16}-HPB in the presence of an exces of albumin are almost identical, shifted approximately 50 nm to a higher wavelength if compared with the free C_{10}-HPB label in solution. The C_{10}-HPB label in the absence of albumin has essentially the

same spectrum as the methyl derivate, indicating that the bulk hydrophobic decyl substituent does not influence the properties of the chromophore and that micelles are formed under the conditions used.

TABLE I

Dependence of the maximum of the charge transfer band of HPB labels in solution and after binding to albumin and to some other globular proteins. The spectra were recorded in 0·15 mol . l^{-1} borate buffer, pH 9·18, at 22°C, in the concentration range 4 . 10^{-6} to 2 . 10^{-5} mol l^{-1}

Label/protein	Molar ratio label/protein	Wavelength nm	Difference to free label, nm
C_1-HPB/aqueous buffer	—	466	—
C_1-HPB/Albumin	1 : 52	460	−6
C_1-HPB/Albumin	1 : 25	468	2
C_4-HPB in aqueous buffer	—	465	—
C_4-HPB/Albumin	1 : 52	468	3
C_4-HPB/Albumin	1 : 25	472	7
C_{10}-HPB in aqueous buffer	—	467	—
C_{10}-HPB/Albumin	1 : 52	513	46
C_{10}-HPB/Albumin	1 : 25	513	46
C_{10}-HPB/Albumin	1 : 1	506	39
C_{10}-HPB/Albumin[a]	1 : 1	490	23
C_{10}-HPB/Albumin[b]	1 : 1	478	11
C_{16}-HPB/Albumin	1 : 52	515	48
C_{16}-HPB/Albumin	1 : 25	514	47
C_{10}-HPB/Lysozyme	1 : 1	468	1
C_{10}-HPB/Basic pancreatic trypsin inhib.	1 : 1	468	1
C_{10}-HPB/Trypsinogen	1 : 1	467	0
C_{10}-HPB/Chymotrypsinogen	1 : 1	467	0
C_{10}-HPB/β-Trypsin	1 : 1	468	1
C_{10}-HPB/α-Chymotrypsin	1 : 1	468	1
C_{10}-HPB/Human apohemopexin	1 : 1	468	1
C_{10}-HPB/Ovalbumin	1 : 1	469	2
C_{10}-HPB/Human gamma globulin	1 : 1	472	5
C_{10}-HPB/Ribonuclease A	1 : 1	468	2

[a] Plus 4 moles of palmitate *per* mole of albumin; [b] plus 8 moles of palmitate *per* mole of albumin.

The small broadening of the absorption band of the labels in the presence of albumin as compared with the free labels in the above solvent indicates that the environment of the bound labels is quite unique.

The other proteins tested (Table I) do not exhibit such effects. For most of the proteins the shift was less than 1 nm, *i.e.* within experimental error.

Competitive Binding of Palmitate

About one third of the label is free in solution, when molar ratio albumin C_{10}-HPB is 1, this brings about an average shorter wavelength of the maximum than found for higher albumin concentrations (Fig. 1, Table I). The position of the maximum is shifted by additional 15 nm to shorter wavelengths if 4 mol of palmitate is added *per* mol of albumin. At a larger palmitate excess (8 mol/mol of albumin) a further drop was observed: the maximum is only 469 nm indicating a competition of the binding of C_{10}-HPB and palmitate and the release of the label to the solution at higher palmitate–albumin ratios (Table I, Fig. 1).

Reaction of 1-(δ-Bromobutyl)-4-(3-ethoxystyryl)pyridinium Bromide with Albumin

The nucleophilic groups of albumin were modified by 1-(δ-bromobutyl)-4-(3-ethoxystyryl)pyridinium bromide (Br-HPB). The dependence of the number of modified

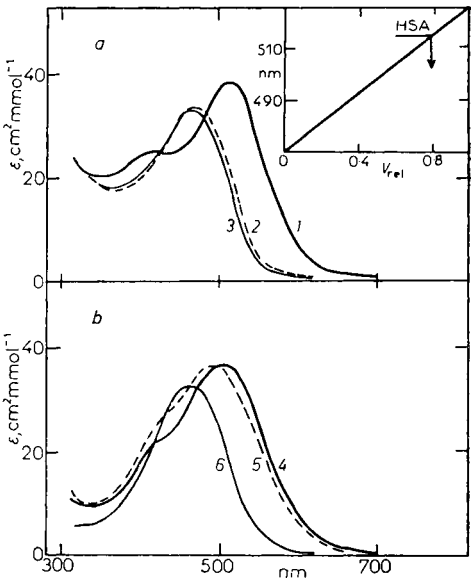


FIG. 1

Spectra of free labels and labels bound to albumin. *1* C_{10}-HPB and C_{16}-HPB label (identical spectra). *2* C_4-HPB and *3* C_1-HPB label in the presence of a 25-fold excess of albumin. *4* C_{10}-HPB label at the same molar concentration of albumin. *5* C_{10}-HPB label under the same conditions as in the case of *4* except that 4 moles of palmitate were added *per* mol of albumin. *6* C_{10}-HPB free label. The spectra were recorded in borate buffer (c 0·15 mol l^{-1}) at 22°C. Insert: Shift of the wavelenght of the maximum of the CT band of the label as a function of the fraction volume of methanol in water. Data were taken from ref.[19]. The position of the maximum of the label bound to albumin is indicated by an arrow

residues of albumin and the dependence of the wavelength of the charge-transfer band on pH of the reaction are shown in Fig. 2. 22 and 54 residues of albumin were modified at pH 7 and pH 10, respectively; while at the beginning of the reaction at pH 5 only 20 molecules of the label are bound to the most reactive groups of albumin. The position of the maximum of the charge-transfer band (476 nm) indicates that the environment of these groups is hydrophilic. As the reaction proceeds the environment of the attached label becomes less polar, as evidenced by a gradual shift of the maximum to higher wavelength.

Chromatographic Experiments

Hydrophobic chromatography on two different supports (Octyl-Sepharose and Spheron) has been used for the investigation of the size and accessibility of the hydrophobic areas on the surface of the albumin molecule.

As evidenced by chromatographic experiments on Octyl-Sepharose (Fig. 3) and Spheron (Fig. 4), human serum albumin has hydrophobic areas localized in the crevices of its molecule.

Albumin was retained on the Octyl-Sepharose column, its elution started when methanol content exceeded 25% (v/v). The distribution coefficient K_D changes from 0·1 to 0·3, if the concentration of methanol is reduced from 40 to 27·5%, but a drastic change occurs in the 27·5 to 25%, range where albumin is not eluted and K_D reaches infinity. The dependences of the recoveries of albumin and defatted albumin on the composition of the elution buffer are shown in Fig. 3. The recovery of defatted albumin is lower, and the dependence on the buffer composition is steeper than that of albumin containing fatty acids.

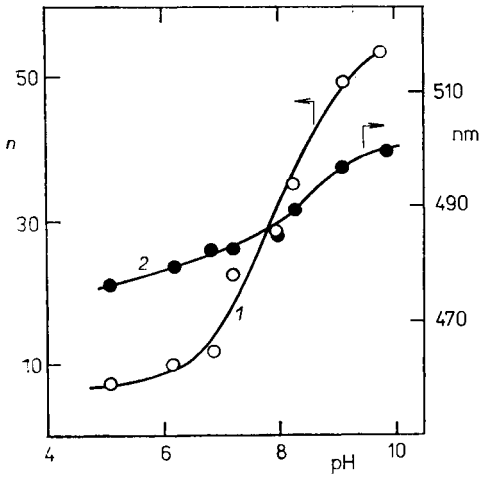


FIG. 2

Reaction of the Br-HPB label with albumin at different pH-values. *1* Dependence of the number of residues of albumin modified by Br-HPB on pH, *2* dependence of the maximum of the CT band of the bound label on the pH of reaction mixture. See Experimental for the conditions

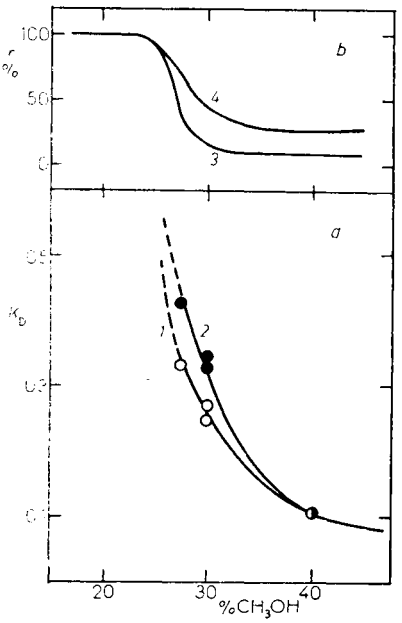


FIG. 3

Chromatographic behavior of albumin on Octyl-Sepharose column. *a* The dependence of the distribution coefficient K_D and *b* retention r of albumin (*1*, *3*) and defatted albumin (*2*, *4*) on the composition of the elution buffer. $K_D = (V_e - V_0) \cdot V_t^{-1}$, V_e — elution volume, V_0 — elution volume in the absence of hydrophobic interactions corresponding to the gel permeation elution volume, V_t — total bed volume. For other conditions see Experimental. 0·05 mol l^{-1} phosphate buffer, pH 5·50, containing the given concentration of methanol

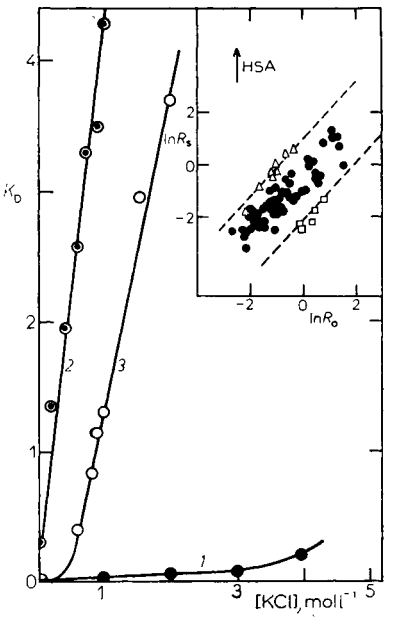


FIG. 4

Chromatographic behavior of albumin on Spheron. Dependence of the distribution coefficient K_D on the concentration of KCl in the elution buffer for *1* human serum albumin, *2* lysozyme, and *3* chymotrypsinogen. 0·05 mol l^{-1} phosphate buffer pH 5·5, 20°C. Insert: Correlation of the retention of some globular proteins during hydrophobic chromatography on Octyl-Sepharose R_o and Spheron R_s under different conditions. Triangles: α-chymotrypsin; full circles: ovalbumin, lysozyme, ribonuclease, chymotrypsinogen, trypsinogen, α-trypsin, β-trypsin, DIP-trypsin, DIP-chymotrypsin, β-chain of oxidized insulin, cow colostrum trypsin inhibitor, and four trypsin isoinhibitors from bull seminal plasma; squares: basic pancreatic trypsin inhibitor. 0·05 mol l^{-1} phosphate buffer, pH 5·5 or 7·3 containing various concentrations of NaCl, KCl, Na_2SO_4, $(NH_4)_2SO_4$, and KBr, 20°C, $R = (V_e - V_0) \cdot V_{solv}^{-1}$, V_{solv} — elution volume of the solvent, for other symbols see Fig. 3. (For the Octyl-Sepharose column $K_D = R$)

Most of the other globular proteins studied are eluted from Octyl-Sepharose columns by aqueous buffers without polarity-reducing organic solvents or detergents. Their retention is over a wide range proportional to the salt concentration of the elution buffer (Table II).

In comparison with Octyl-Sepharose a different chromatographic behaviour of albumin was observed on Spheron P 300 (Fig. 4). While the retention of many other proteins on Spheron and Octyl-Sepharose is comparable, albumin is retained on Octyl-Sepharose, but it is not retained at neutral pH on Spheron even at a salt concentration up to 3 M-KCl. The dependence of the retention of albumin on Spheron on the concentration of KCl in the elution buffer was compared to that of lysozyme and chymotrypsinogen. The latter proteins exhibit a steeper dependence of K_D than albumin. The recovery of lysozyme and chymotrypsinogen from the Spheron column is much less influenced by the strength of the interaction with the support. Albumin

TABLE II

Comparison of the retention for human serum albumin and several other proteins on Octyl-Sepharose and Spheron at various concentrations of NaCl. *A* — Spheron, *B* — Octyl-Sepharose. For conditions and symbols see legends to Figs 3 and 4

Protein	Concentration of NaCl, mol l^{-1}							
	0·5		1·0		1·5		2·0	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
Albumin	0·03	10	0·02	10	0·02	10	0·06	10
Ribonuclease	0·12	0·07	0·13	0·07	0·16	0·12	0·27	0·25
Basic pancreatic trypsin inhibitor	0·9	0·1	1·4	0·18	2·6	0·34	—	—
Bull seminal trypsin inhibitor I	2·0	0·3	5·1	0·45	—	—	—	—
Bull seminal trypsin inhibitor II	0·07	0·09	0·12	0·03	0·16	0·08	—	0·12
Cow colostrum trypsin inhibitor	0·13	0·16	0·12	0·11	0·19	0·18	0·26	0·28
Chymotrypsin	0·26	0·8	0·47	3·8	1·0	—	—	—
Chymotrypsinogen	1·2	1·1	2·8	3·8	8·9	—	—	—
Trypsin	0·4	0·29	1·6	0·85	3·0	—	—	—
Lysozym	4·1	2·8	—	—	—	—	—	—

on the other hand shows on both supports a sharp decrease of recovery with a small increase of the K_D value.

The correlation of the retention of different globular proteins on Octyl-Sepharose and Spheron under different conditions is shown on the inset in Fig. 4.

The difference in the chromatographic behaviour of albumin on Octyl-Sepharose and Spheron is supposedly caused by the "hidden" (pocket) nature of the hydrophobic sites on the surface of the albumin molecule. Experiments designed to verify this assumption are summarized in Table III. Defatted albumin was labeled by C_{16}-HPB. The competition of the binding of the label by albumin and by Spheron was investigated at neutral pH; at pH 3 and after the acidified solution had been brought back to neutral pH. Despite of the strong sorption capacity of Spheron for the C_{16}-HPB label at neutral pH, the label as well as albumin are in solution. At pH 3 almost complete sorption of both albumin and the label to the resin occurs. If the mixture is brought back to pH 7·9, the protein is released but the C_{16}-HPB label is still preferentially bound to Spheron, even after a sufficient time to reach new equilibrium (Table III).

This phenomenon is more pronounced during chromatographic experiments, where the ratio of the amount of the sorbent to the applied quantity of labeled albumin is of several orders of magnitudes higher. When 0·02 mg of albumin labeled with C_{16}-HPB (2 : 1) was applied onto a 7·6 ml column of Spheron P 300 at neutral pH, the ratio of the absorbance at 280 and 239 nm of the effluent was approximately

TABLE III

Binding of the C_{16}-HPB label to albumin and Spheron *A*. Defatted albumin C_{16}-HPB label 1 : 2 mixture in 0·05 mol l^{-1} phosphate buffer pH 5·50, *B* the same as in *A* except that 0·1 g of Spheron P 300 was added *per* 2 ml of solution (the concentration increase is caused by the water uptake by the resin), *C* sample *B* when brought to pH 2·95 by 0·5 mol l^{-1} HCl, *D* sample *B* when brought to pH 2·95 for 30 min, than back to pH 7·89 by 0·5 mol l^{-1} NaOH and measured after 12 min. The concentrations were determined spectrophotometrically at pH 5·50 and 280 and 389 nm

Experiment	pH	Concentration in solution, mol l^{-1}		Molar ratio of C_{16}-HPB/albumin in solution
		albumin	C_{16}-HPB	
A	5·50	$3{\cdot}0 \cdot 10^{-5}$	$6 \cdot 10^{-5}$	2
B	5·50	$3{\cdot}6 \cdot 10^{-5}$	$8{\cdot}1 \cdot 10^{-5}$	2·2
C	2·95	$1{\cdot}6 \cdot 10^{-6}$	$8 \cdot 10^{-7}$	5
D	7·89	$3 \cdot 10^{-5}$	$2{\cdot}1 \cdot 10^{-6}$	0·07

the same as with the original sample. The C_{16}-HPB label alone in the absence of albumin is strongly adsorbed on the top of the column and is not eluted until alcohol is used.

DISCUSSION

Solvatochromic HPB labels substituted with nonpolar decyl or hexadecyl group are strongly bound to the hydrophobic binding site of albumin. The polarity of the microenvironment of the bound labels is lower than that of the bulk solution, corresponding approximately to 80% methanol or 60% ethanol in water[26]. The large red shift accompanying the binding to albumin, not observed with other globular proteins used in this study, reflects changes of water structure and water solvation ability in the neighborhood of a large hydrophobic domain. Other proteins of known X-ray structure, like lysozyme[27], β-trypsin[28], trypsinogen[29], α-chymotrypsin[30], chymotrypsinogen[31], or basic pancreatic trypsin inhibitor[32] have hydrophobic areas or clusters of nonpolar residues exposed to the solvent[33–35]. In our experiments none of these proteins, however, exhibited such large shifts as those observed with albumin. The hydrophobic patches of albumin must be therefore more extensive and localized in crevices or holes. The size of the hole enables the binding of ten decyl but only four hexadecyl substituted label molecules. The binding site of albumin site of albumin for C_{10}-HPB and C_{16}-HPB are the same as those for fatty acids because the first label is released into solution by palmitate. The binding affinity of albumin for the alkyl substituted labels is of the order of association constants determined for other amphiphilic compounds with nonpolar substituents of the same size[35–40]. The hydrocarbon tail is responsible for the binding strength, the chromophore groups contribute only weakly to the binding, as the interaction of the methylsubstituted label C_1-HPB with albumin is by several orders of magnitude weaker than that observed with C_{16}-HPB and C_{10}-HPB. The differences in the values of the association constants of the C_{10}-HPB label for albumin at pH 5·5, determined in the ultracentrifuge, and at pH 9·2, determined by spectrophotometric titration, cannot be completely ascribed to the different techniques used. Defatted albumin has a broader N–F transition pH-range[41]; at pH 5·5 the defatted albumin is partially destabilized and that probably influences its binding ability.

Reaction of Br-HPB with albumin at lower pH preferentially directed to the residues with a stronger nucleophilic character located on the surface of the molecule.

Brown has suggested that some unsually reactive residues of albumin are located near the mouth of the hypothetical binding hole[2].

The environment of the most reactive surface nucleophilic groups is predominantly hydrophilic, because the red shift of the CT band of the label when about ten or twenty residues are modified is three or four times smaller than when it is bound to the hydrophobic site. As the reaction proceeds the environment of the label at-

tached to albumin becomes less polar, indicating that some hindered or less exposed residues or residues close to the hydrophobic domain are also modified.

The results obtained with the spectral labels are supported by hydrophobic chromatography (Table II). The retention of the indiviual proteins at different conditions on two supports (*i.e.* on Octyl-Sepharose and Spheron) is proportional, but different proteins exhibit different slopes. The flexibility of the ligand and the spacer Octyl--Sepharose can explain the interaction of both the nonpolar groups of proteins exposed to an external environment as well as groups localized in crevices (holes). Spheron on the other hand, is a highly cross-linked macroporous resin[42] which preferentially interacts with nonpolar residues on the surface of proteins, exposed to an external environment. The hydrophobic binding sites of Spheron are formed by a highly crosslinked polymer backbone which has a limited flexibility and accessibility and which cannot interact with nonpolar groups localized in crevices (holes or pockets).

Spheron binds more strongly low molecular weight solutes as well as most proteins than Octyl-Sepharose. At neutral pH Spheron does not bind albumin, indicating that in the neutral pH-range (pH 5–9) the hydrophobic binding sites of albumin are not accessible to the resin because the binding sites are localized in holes. A very weak interaction albumin with Spheron up to 3M-KCl reveals that the outer surface of albumin is more hydrophilic than that of most of other proteins. Very few nonpolar side chains are placed outside the holes on the exposed surface. These results confirm the picture of the structure of the albumin molecule presented by Brown[2]. At pH 3 (upon N–F transition) albumin looses the label and bounds to the support; its hiden hydrophobic sites become exposed. Such structure changes accompanying the N–F transition can be expected because of the extreme solubility of the N-form at neutral pH nd the virtual insolubility of the F-form at pH lower than 4 (refs[2,43]).

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