ELSEVIER

Journal of Chromatography B, 666 (1995) 21–31

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Influence of salts on protein interactions at interfaces of amphiphilic polymers and adsorbents☆

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First received 15 June 1994; revised manuscript received 22 November 1994; accepted 28 November 1994

Abstract

The protein-binding capacity of two different amphiphilic adsorbents was investigated to determine the effect of solvent additives on the binding of proteins in hydrophobic-interaction chromatography. There was no simple correlation between binding capacity and the lyotropic series such as those suggested by the two different theories proposed by Arakawa and Narhi and Melander and Horváth. Proteins are known to be dynamic flexible objects which continuously undergo changes in conformation and which may well be influenced by chaotropic salts. Are conformational changes of proteins at interfaces an important parameter involved in protein interactions with amphiphilic polymers and adsorbents? In an attempt to answer this question, the reactivity of the thiol group in human serum albumin (HSA) toward N-ethyl-3-(2-pyridyldisulfanyl)propionamide dextran was used as a model system to evaluate its correlation with the lyotropic series. The results indicate that the thiol–disulfide exchange reaction at interfaces of amphiphilic polymers is influenced by the type of salt used.

1. Introduction

When proteins interact with surfaces of amphiphilic nature different more or less well defined factors contribute to this interactive process. The hydrophobic effect is one of these contributing factors manifested by the very low solubility of nonpolar substances in water and the tendency of nonpolar solutes to form aggregates in aqueous solution. Two theories have been developed to account for this effect. One focuses on the structure of water [1]. The second approach is the cavity model [2]. In both theories the hydrophobic effect is driven either by entropy [3] or enthalpy [4,5].

In addition to the hydrophobic effect, the charge-transfer concept introduced by Mulliken [6] describes another type of specific interactions which has been suggested to play a significant role when aromatic structures are present [7].

An other important contributing factor is the influence of salt additives on the adsorption of proteins onto hydrophobic surfaces. This has been evaluated by Melander and Horváth [8] and later by Fausnaugh et al. [9], Arakawa and Narhi [10] and Sokolowski [11]. Also covalent chromatography [12] and, in some cases, immobilized-metal affinity chromatography (IMAC) [13] have been found to be strongly

☆ Paper presented at the *Bio-chromatography and Bio-engineering Symposium, Vth Symposium of the European Society for Bio-Chromatography, May 17–19, 1994, Nancy, France.*

0378-4347/95/$09.50
SSDI 0378-4347(94)00550-8

influenced by salt additives in the solvent. In the case of covalent chromatography the increased adsorption capacity was shown to be caused by conformational changes in the thiol-containing protein human serum albumin (HSA) upon contact with the surface [12].

In an attempt to further investigate some of the factors influencing the specificity and the capacity of the adsorption of proteins, at least seven different types of salts were used in combination with an amphiphilic adsorbent.

2. Experimental

2.1. Chemicals

All salts were purchased from Fluka (Buchs, Germany), and 2-chloroethylamine monohydrochloride from Aldrich (Milwaukee, WI, USA).

Sodium borohydride, $Na_2H_2PO_4 \cdot 1H_2O$, ethylene glycol and HCl were obtained from Merck (Darmstadt, Germany). Trizma base, HSA and dithioerythreitol were purchased from Sigma (St. Louis, MO, USA) and NaOH from EKA Kemi (Surte, Sweden).

SPDP [N-succinimidyl - 3 - (2 - pyridyldisulphanyl)propionate], Octyl-Sepharose, dextran and PD-10 columns were kindly supplied by Pharmacia (Uppsala, Sweden).

2.2. Samples

Serum samples (mixed from 50 patients to obtain a serum pool) were purchased from the University Hospital (Uppsala, Sweden). Dialysis tubing and agarose were purchased from Kebo Lab. (Stockholm, Sweden). Specific antibodies against serum proteins were a gift from C.L. Laurell, Malmö Hospital (Malmö, Sweden).

2.3. Determination of dynamic capacity

Serum was diluted with 0.1 *M* Tris buffer (pH 7.5) and potassium sulphate was added to obtain a final protein concentration of 13.6 mg protein per ml in the presence of 0.5 *M* potassium sulphate. A column (3 × 1 cm I.D.) filled with the respective adsorbent was equilibrated with 0.5 *M* potassium sulphate–0.1 *M* Tris buffer at a flow-rate of 0.64 ml/min. The protein solution was loaded onto the column at the same flow-rate until the protein concentration in the eluate from the column became the same as that in the applied sample.

2.4. Determination of salt-dependent adsorption capacity

In order to achieve high reproducibility during the chromatography, an optimized program for adsorption, desorption and rinsing of the adsorbent was executed by means of a programable FPLC system. Serum was diluted with 0.1 *M* Tris buffer (pH 7.5) to attain a protein concentration of 13.6 mg protein/ml in the presence of the salt to be studied. A column (3 × 1 cm I.D.) packed with the desired adsorbent was equilibrated with the salt dissolved in 0.1 *M* Tris buffer. At a flow-rate of 0.64 ml/min, a 1-ml sample was loaded onto the column followed by 15 ml of the salt solution in 0.1 *M* Tris buffer. A 10-ml volume of 0.1 *M* Tris buffer was passed through the column at a flow-rate of 0.64 ml/min. Finally, the gel was rinsed with 0.1 *M* NaOH when mercaptopyridine agarose was used and with 20% (v/v) ethanol in deionized water for the other adsorbent. The above described procedure was repeated at 75% and 50% of the original salt concentrations. In some cases even 25, 12.5 and 6.25% of the original salt concentrations were used.

Elemental analyses were performed with an automated nitrogen–sulfur analyzer NA 1500 Carlo Erba Strumentazione (Rodans, Milan, Italy) by Erik Forsman (Dept. of Anal. Chem., Uppsala University). Chromatography was performed using an FPLC system equipped with a programable LCC 500 control unit. Spectrophotometric analyses were performed with a Shimazu UV-160 A spectrophotometer. Immunodiffusional analyses were performed in 1% agarose gel.

2.5. Preparation of N-ethyl-3-(2-pyridyldisulphanyl)propionamide dextran (Py-S_2 dextran)

Amination of dextran

Dextran [$\overline{M}_w$ (light scattering): 10 500; $\overline{M}_n$ (end group analysis) : 6500], 1 g, was mixed with 40 ml of distilled water, 9.8 g NaOH and 0.4 g $NaBH_4$. Four grams of chloroethylamine were added to the stirred solution. After a 15-h reaction time at 60°C, the solution was neutralized with 5 *M* acetic acid. The solution was centrifuged and the supernatant was withdrawn and dialyzed against distilled water for 48 h.

Functionalization of aminodextran

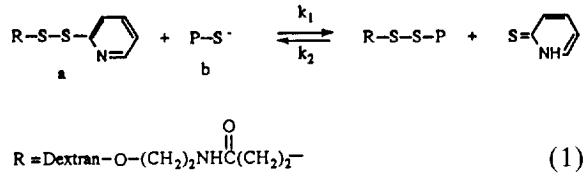
Aminated freeze-dried dextran, 0.5 g, was dissolved in 60 ml of 50 m*M* sodium borate (pH 9.0), containing 30 ml of ethanol. SPDP, 200 mg, was added to the solution and the reaction was continued at room temperature for 2 h followed by dialysis for 24 h against distilled water–ethanol (4:1, v/v). After freeze-drying of the dialyzed solution containing the derivatized dextran, elemental analysis gave a value of 162 μmol S/g.

2.6. Reaction of HSA with Py-S_2 dextran in different salt solutions

An 800-μl volume of 0.1 *M* Tris-HCl (pH 7.5) was added to the reference cuvette and the sample cuvette. A 100-μl volume of Py-S_2 dextran ($1.62 \cdot 10^{-3}$ *M*, S-S-pyridine groups) in 0.1 *M* Tris (pH 7.5) was added to the reference cuvette and the sample cuvette. Finally, 100 μl of HSA (dissolved in 0.1 *M* Tris, pH 7.5, $0.92 \cdot 10^{-4}$ *M*) was added to the sample cuvette and the release of 2-thiopyridone was registered at 343 nm during the first 130 s. This procedure was repeated with 2 *M* $(NH_4)_2SO_4$, 2 *M* NH_4Cl, 2 *M* CH_3COONH_4, 2 *M* $(NH_4)_2PO_4$, 2 *M* NH_4F and 2 *M* NH_4Br, all dissolved in 0.1 *M* Tris-HCl with the pH adjusted to pH 7.5.

2.7. Calculation of the rate constant for the reaction of HSA with Py-S_2 dextran in different salt solutions

The rate constants were determined for the reaction:



R = Dextran–O–$(CH_2)_2$NHC(=O)$(CH_2)_2$– (1)

where $[a_0] = 1.62 \cdot 10^{-4}$ *M* (initial concentration for Py-S_2 dextran) and $[b_0] = 9.2 \cdot 10^{-6}$ *M* (initial concentration for native HSA) and P = protein.

The rate reaction can be approximated by:

$$\frac{d[b]}{dt} = k_1 \cdot [a] \cdot [b] \quad (2)$$

since the reversed reaction can be neglected for the short reaction times used. The reaction according to Eq. (1) has been shown to be second order [8]. The data obtained were treated according to the procedure described in Ref. [12].

3. Results

3.1. Dynamic capacities

The maximum dynamic capacities in potassium sulphate solutions for octyl- and mercaptopyridine derivatized agarose were found to be respectively 28.4 and 21.0 mg protein per ml of gel.

3.2. Salts in the elutropic series. Effect on protein adsorption capacity

In an attempt to investigate the effect of different combinations of anions and cations in promoting protein adsorption, octyl- and mercaptopyridine-derivatized agaroses were selected as adsorbents, with an optimal degree of substi-

tution used during the capacity studies [14]. The salt-dependent adsorption capacity (SAC) is studied in the following and is defined as the percentage of the adsorbed protein that can be released from the adsorbent by deleting salt from the elution buffer. Fifteen different types of salts were used in combination with two types of adsorbents and at least three salt concentrations in an attempt to investigate the order among different salts with respect to adsorption capacity and the influence of the adsorbent on this order.

Variable anions, constant cations

For ammonium, sodium and potassium ions in combination with different anions, sulphate ions promote protein adsorption more than do chloride and acetate ions on both mercapto- and octyl-derivatized agarose (see Figs. 1a,b–3a,b).

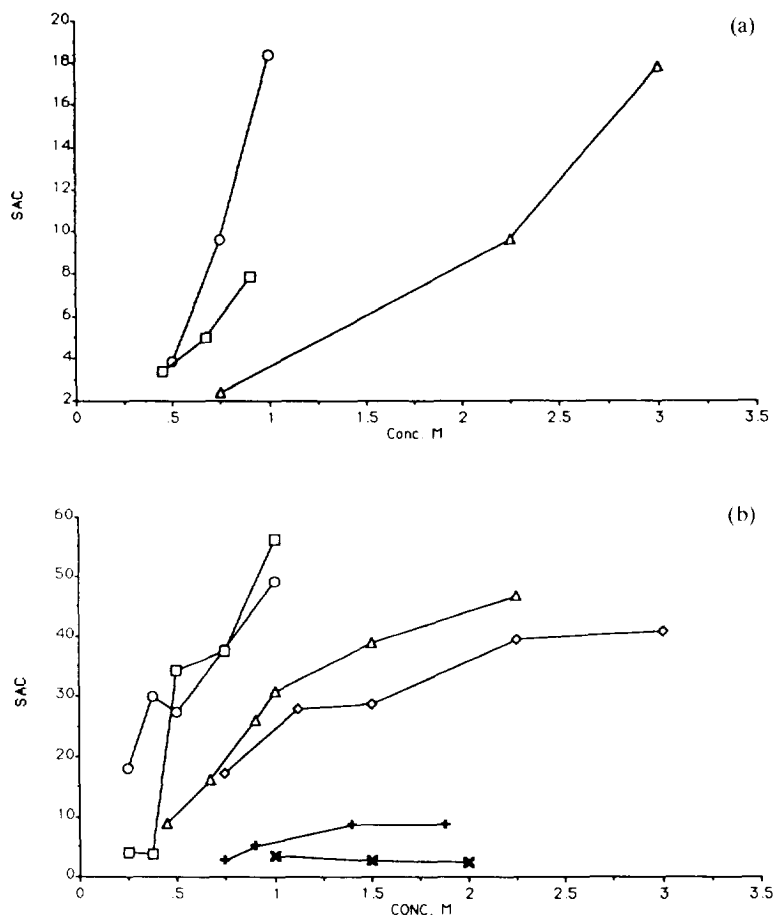


Fig. 1. (a) Percent of material eluted (SAC) from an Octyl-Sepharose column (3 × 1 cm I.D.) upon deleting (○) ammonium sulphate, (□) ammonium chloride, (△) ammonium acetate from a 0.1 *M* Tris buffer (pH 7.5). The protein solution was loaded onto the column in the presence of the respective salt and salt concentration at a flow-rate of 0.64 ml/min. (b) Percent of material eluted (SAC) from a mercaptopyridine-derivatized agarose column (3 × 1 cm I.D.) upon deleting (○) ammonium sulphate, (+) ammonium chloride, (×) ammonium bromide, (□) ammonium phosphate, (△) ammonium fluoride, (◇) ammonium acetate from a 0.1 *M* Tris buffer (pH 7.5). The protein solution was loaded onto the column in the presence of the respective salt and salt concentration at a flow-rate of 0.64 ml/min.

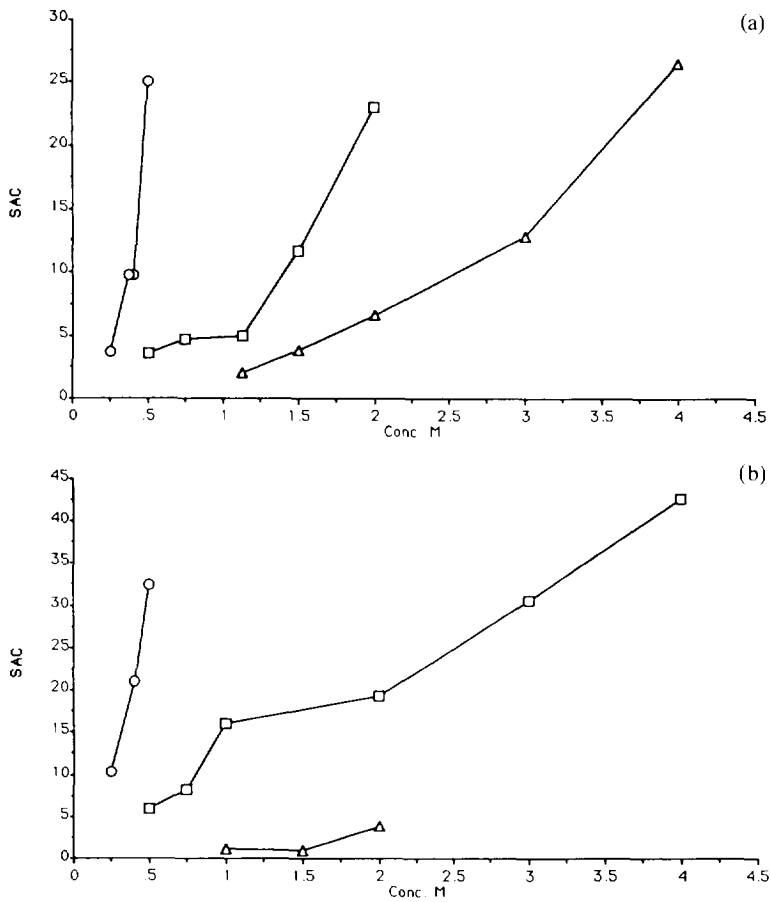


Fig. 2. (a) As Fig. 1a except that following salts were used: (○) potassium sulphate, (△) potassium chloride, (□) potassium acetate. (b) As Fig. 1b except that following salts were used: (○) potassium sulphate, (□) potassium chloride, (△) potassium bromide.

The relation between chloride and acetate ions in promoting protein adsorption depends both on the type of adsorbent and the type of cation used. When ammonium ions are used in combination with octyl-derivatized agarose, chloride ions promote protein adsorption stronger than does acetate. On the other hand, when sodium ions are used in combination with the same adsorbent the order is reversed. Upon changing from octyl- to mercaptopyridine-derivatized agarose in combination with ammonium ions the order of chloride and acetate is reversed. When different ammonium salts were used in combination with the mercaptopyridine-derivatized adsorbent, fluoride ions were found to promote adsorption more effectively than acetate, chloride and bromide ions.

Variable cations, constant anions

When chloride ions were used in combination with Octyl-Sepharose and different cations, ammonium chloride was found to be more effective in promoting protein adsorption than sodium. Sodium, on the other hand, has the same efficiency as potassium (see Fig. 4a). Upon changing to the mercaptopyridine-derivatized adsor-

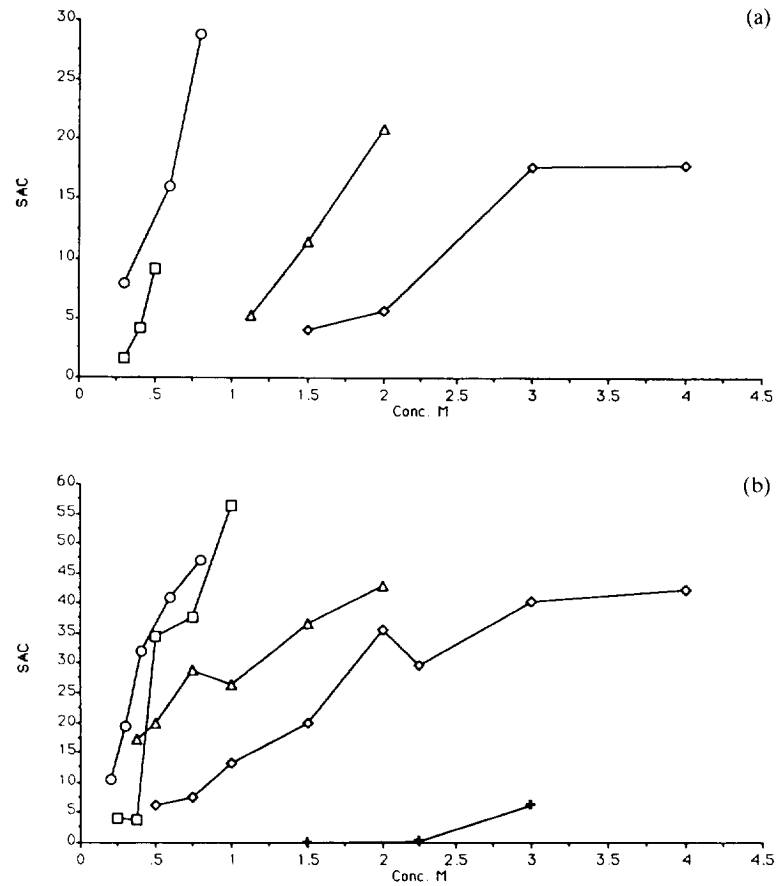


Fig. 3. (a) As Fig. 1a except that following salts were used: (◇) sodium chloride, (□) sodium sulphate, (△) sodium acetate, (○) sodium phosphate. (b) As Fig. 1b except that following salts were used: (○) sodium sulphate, (+) sodium bromide, (◇) sodium chloride, (□) sodium phosphate, (△) sodium acetate.

bent, the opposite results were obtained (see Fig. 4b). When sulphate ions were used instead of chloride, in combination with Octyl-Sepharose, potassium and sodium were the most effective salts. Upon changing the adsorbent from octyl- to mercaptopyridine-derivatized agarose, ammonium sulphate was found to be more effective than both sodium and potassium in the concentration range 0.1–0.5 *M* (see Fig. 5b). A more extensive study of protein adsorption capacity has been conducted on the mercaptopyridine-derivatized adsorbent. In combination with different sulphate salts, ammonium ions are more effective than sodium and potassium in promoting protein adsorption. Magnesium sulphate is the least effective.

3.3. Salts in the elutropic series. Effect on selectivity in protein adsorption

The influence of salts in the elutropic series on the adsorption of seven different serum proteins has been analyzed (see Table 1). Serum was applied to octyl- or mercaptopyridine-derivatized agarose as described in the Experimental section. The proteins that were adsorbed in the presence of different types of salts and which eluted upon deleting salt from the elution buffer were identified by immunodiffusion by use of specific antibodies against the serum proteins of interest. According to Table 2, orosomucoid was only detected in the eluate when ammonium acetate was used in combination with mercap-

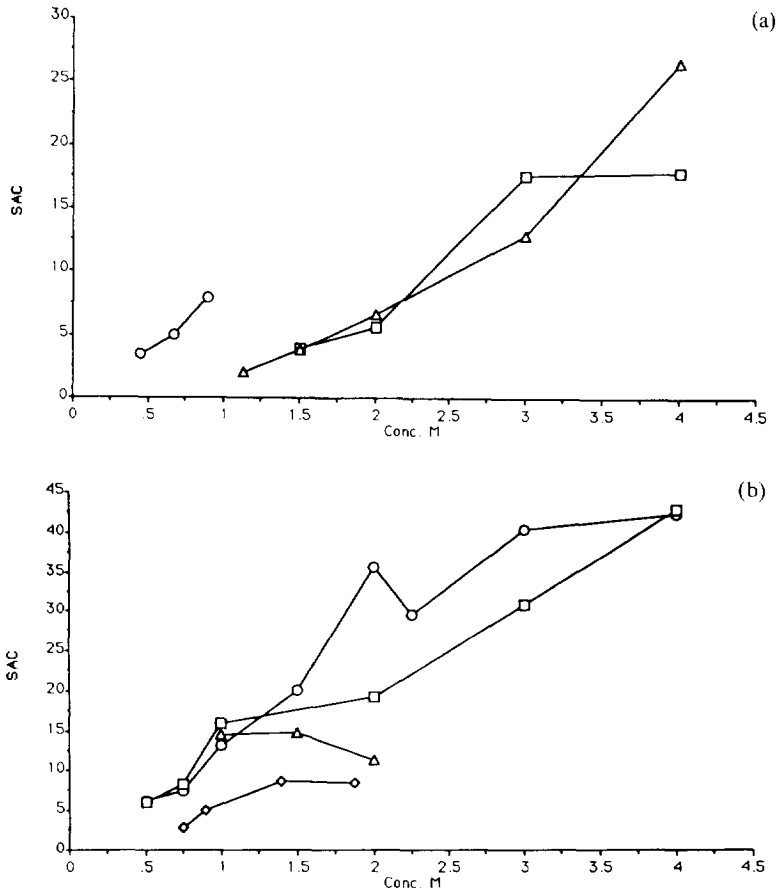


Fig. 4. (a) As Fig. 1a except that following salts were used: (△) sodium chloride, (□) potassium chloride, (○) ammonium chloride. (b) As Fig. 1b except that following salts were used: (○) sodium chloride, (□) potassium chloride, (△) magnesium chloride, (◇) ammonium chloride.

topyridine-derivatized agarose. With all other combinations of salts no orosomucoid was detected. On Octyl-Sepharose, orosomucoid was detected when potassium chloride was used.

IgA was found in the eluate when sodium chloride, potassium chloride or ammonium acetate were used in combination with Octyl-Sepharose. Upon changing the adsorbent to mercaptopyridine-derivatized agarose, IgA was found in the eluate in combination with all of the investigated salts except with ammonium chloride (see Table 1).

IgG became adsorbed both onto Octyl-Sepharose and mercaptopyridine-derivatized agarose independently of the type of salt used. HSA also adsorbs to Octyl-Sepharose in combination with all of the salts investigated. Upon changing the adsorbent to mercaptopyridine-derivatized agarose, the effect of the type of salt was important, since HSA was detected in the eluates with all salts except potassium sulphate and ammonium chloride (see Table 2).

Adsorption of transferrin to Octyl-Sepharose was also found to be strongly influenced by the type of salt used. Transferrin was not detected in the eluate when ammonium chloride and sodium chloride were used (see Table 1). When mercaptopyridine-derivatized agarose was used no transferrin was detected in the eluate for the investigated salts.

α_2-Macroglobulin is adsorbed to octyl- and mercaptopyridine-derivatized agarose in the

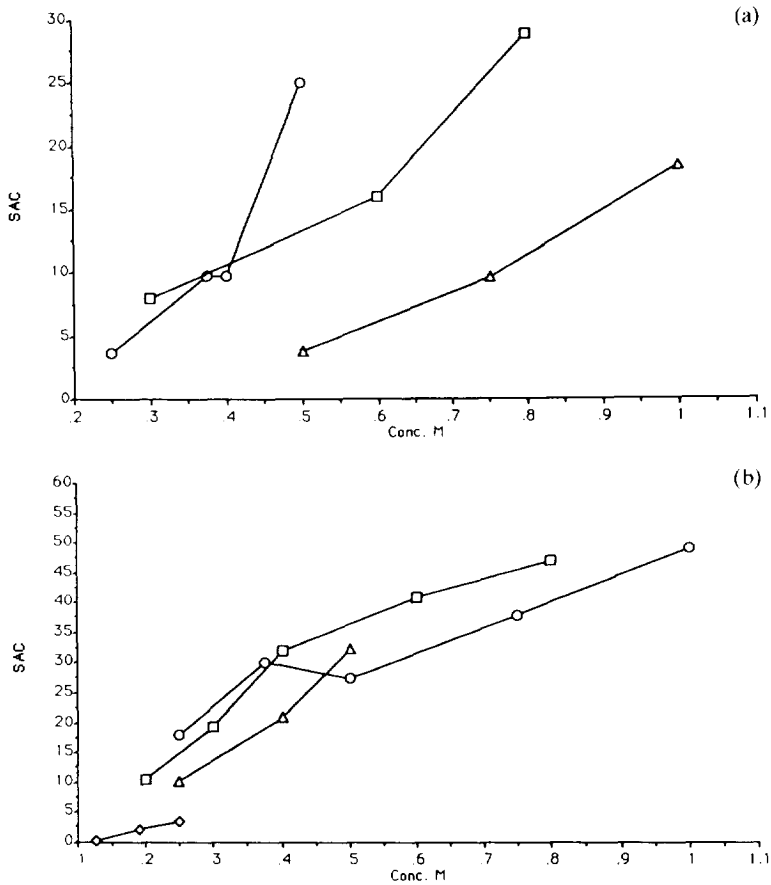


Fig. 5. (a) As Fig. 1a except that following salts were used: (○) potassium sulphate, (△) ammonium sulphate, (□) sodium sulphate. (b) As Fig. 1b except that following salts were used: (□) sodium sulphate, (△) potassium sulphate, (○) ammonium sulphate, (◇) magnesium sulphate.

Table 1
Identification of proteins by immunodiffusion in materials desorbed from Octyl-Sepharose after exclusion of respective salt from the buffers

Salt	Conc.	1	2	3	4	5	6	7
K_2SO_4	0.5 *M*	+		+		+	+	
NH_4Ac	2.25 *M*	+	–	+	+	+	+	
NH_4Cl	0.9 *M*	–	–	+	–	+	–	
NaCl	3 *M*	–		+	+	+	–	
KCl	3 *M*	+	+	+	+	+	+	+

1 = α_2-Macroglobulin; 2 = orosomucoid; 3 = IgG; 4 = IgA; 5 = albumin; 6 = transferrin; 7 = ceruloplasmin.
+ = Protein detected in the eluate; – = protein not detected in the eluate.

Table 2
Identification of proteins by immunodiffusion in materials desorbed from mercaptopyridine-derivatized agarose after exclusion of respective salt from the buffers

Salt	Conc.	1	2	3	4	5	6	7
K_2SO_4	0.5 *M*	+	–	+	+	–	–	+
NH_4Ac	2.25 *M*	+	+	+	+	+	–	+
NH_4Cl	0.9 *M*	–	–	+	–	–	–	+
NaCl	3 *M*	–	–	+	+	+	–	+
KCl	3 *M*	+	–	+	+	+	–	–

1 = α_2-Macroglobulin; 2 = orosomucoid; 3 = IgG; 4 = IgA; 5 = albumin; 6 = transferrin; 7 = ceruloplasmin.
+ = Protein detected in the eluate; – = protein not detected in the eluate.

presence of all of the investigated salts except ammonium chloride and sodium chloride.

Ceruloplasmin was found to be adsorbed to Octyl-Sepharose only in the presence of potassium chloride. In the case of mercaptopyridine-derivatized agarose the effect of salts on ceruloplasmin adsorption is totally opposite, since this protein is adsorbed in the presence of all of the salts investigated except potassium chloride.

3.4. Reaction of HSA with Py-S_2 dextran in different salt solutions

The reaction between the native thiol group in HSA and the pyridyl-disulfide group in Py-S_2 dextran was determined at room temperature and pH 7.5 in the presence of the respective salts at a concentration of 1.6 *M*. Three independent measurements during the first 130 s gave the average second-order rate constant according to Table 3. The highest second-order rate constant was observed in the presence of ammonium fluoride, followed by ammonium chloride and ammonium phosphate at nearly the same reaction rates. A lower reaction rate was observed in the presence of ammonium sulphate compared to ammonium chloride and ammonium phosphate. The reaction rate was about 3-fold lower in the presence of ammonium acetate and more than four times lower in the presence of ammonium bromide as compared to ammonium fluoride.

Table 3
Average apparent second-order rate constants (from three determinations) for the reaction of HSA with Py-S_2 dextran in the presence of 1.6 *M* (final concentration) ammonium salt solutions in 0.1 *M* Tris-HCl at 22°C and pH 7.5

Type of salt	Rate constant ($M^{-1} s^{-1}$) ± S.D.
NH_4Cl	2.29 ± 0.03
NH_4Br	0.61 ± 0.02
NH_4F	2.54 ± 0.05
$(NH_4)_2SO_4$	2.17 ± 0.02
$(NH_4)_3PO_4$	2.26 ± 0.03
NH_4Ac	0.90 ± 0.07

4. Discussion

Proteins in solution are known to switch between different conformations [15]. Some of these switches can be more pronounced in different micro-environments, leading to exposure of certain structures. Despite extensive studies on the folding and unfolding processes the physical basis for those more drastic conformational changes is still not clear, but according to existing dogma protein folding is driven by hydrophobicity. Recently Murphy et al. [16] presented the contradictory statement that: " . . . the liquid hydrocarbon model suggests that hydrophobic interactions always lead to stabilization whereas the analysis of general capacity effects found for globular proteins lead to destabilization".

For interactions between proteins and hydrophobic surfaces different theories have been proposed to explain the effect of solvent additives on the binding of proteins in hydrophobic interaction chromatography. According to Melander and Horváth [8] a salt that affects hydrophobic interactions can be characterized by quantification of its molar surface tension increment, which is a measure of the increase in surface tension caused by the salt. The same paper presents a list of the molar surface tension increments of various salts, where a high value correlates with a more efficient adsorption of proteins to the hydrophobic adsorbent.

The results presented here are contradictory, since ammonium sulphate was found to be more effective in promoting protein adsorption on mercaptopyridine-derivatized agarose than potassium and sodium sulphate, which are more effective than magnesium sulphate. According to Melander and Horváth [8], sodium sulphate has the highest molar surface tension increment, followed by potassium sulphate, ammonium sulphate and the lowest value for magnesium sulphate. Results from studies with different chloride salts also show deviations from what might be expected from the molar surface tension increment values, since magnesium chloride promotes protein adsorption more than sodium chloride according to Melander and Horváth. The opposite result was obtained in this study

when mercaptopyridine-derivatized agarose was used. For all investigated salts SAC increases with increasing salt concentration except for magnesium chloride (see Fig. 4b) and ammonium bromide (see Fig. 1b). One reason for a lower SAC value for these salts with increasing salt concentration may be that the proteins are more strongly bound to the adsorbent as a result of more pronounced conformational changes.

The results obtained from studies on the influence of different cations on protein adsorption in combination with a given anion show that the type of adsorbent strongly influences the salt-promoting order of protein adsorption. The results obtained with mercaptopyridine-derivatized agarose in combination with different chloride salts (see Fig. 4b) show a high correlation with the molar surface tension increment values, but no correlation at all is observed with Octyl-Sepharose. When different sulphate salts are used in combination with Octyl-Sepharose the correlation between the results presented here and the molar surface tension increment values is high, but low in combination with mercaptopyridine-derivatized agarose. All of these contradictory results show that no simple correlation exists between the influence of salts on surface tension and hydrophobic interactions as proposed by Melander and Horváth.

According to the results presented in Tables 1 and 2 the salt strongly influences the type of protein adsorbed to octyl- and mercaptopyridine-derivatized agarose. Why do we have adsorption of a protein to an adsorbent in the presence of one salt but not in the presence of another salt? In an attempt to investigate the influence of different ammonium salt solutions on the interaction between Py-S_2 dextran and native HSA, the second-order rate constant was determined for the thiol–disulphide exchange reaction between the pyridyldisulphide group and the buried thiol group in native HSA. From earlier studies [12], we know that the rate constant is low, 0.57 $M^{-1}\,s^{-1}$ in the presence of 0.1 M Tris at pH 7.5. Upon introducing a thiol group on the native HSA molecule, the rate constant for the same reaction under the same conditions increases with a factor of nearly fifty [12]. This indicates that the native thiol group is buried and is not exposed on the surface. The new thiol group is introduced preferentially on primary amino groups which are exposed on the surface of HSA. In 1.6 M solutions of different ammonium salts, a strong correlation exists between the type of salt used and the rate constant for the thiol–disulphide reaction. These results indicate that the native thiol group becomes more or less exposed in different salt solutions.

These results show a rather chaotic situation at the interfaces of polymers and adsorbents in the presence of salts. If these results are general for all types of proteins the influence of salts and the adsorbent on the conformation of proteins at interfaces will be one possible explanation for the many conflicting and contradictory results often found for hydrophobic interactions, and which are difficult to explain in terms of existing theories.

Acknowledgements

I would like to thank the Swedish Government for Science and Education for financial support. I also thank Dr. D. Eaker for linguistic revision of this paper.

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