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Thiophilic Adsorption: A Comparison of Model Protein Behavior†

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ABSTRACT: A newly recognized type of protein–ligand interaction phenomenon has resulted in the preparation of simple, nonionic, and highly specific gel derivatives for selective adsorption chromatography. The essential structure of the immobilized ligand can be represented as agarose–$CH_2CH_2SO_2CH_2CH_2SCH_2CH_2OH$, which was prepared by using mercaptoethanol to derivatize [0.9–1.0 mmol (g of dry gel)$^{-1}$] divinyl sulfone activated agarose (thiophilic or T-gel). Proteins interacting with this ligand are provisionally termed "thiophilic" to recognize their affinity for the definitive sulfone–thioether constituents. To better understand the experimental variables affecting adsorption efficiency and selectivity, several well-characterized proteins with diverse physicochemical features have been evaluated for thiophilic properties. Thiophilic interaction chromatography was investigated as a function of pH as well as the type and concentration of water-structure-forming salts required to promote adsorption. The model proteins characterized varied distinctly in their individual thiophilic affinities. At acidic pH values, a salt-independent adsorption process was observed. Furthermore, a minimum in the salt-promoted thiophilic adsorption tendency at pH 5–6 was found, with varying magnitude, for each of the model proteins evaluated. Recovery of adsorbed proteins routinely varied from 90% to 100%. There does not appear as yet to be any easily recognized physicochemical property associated with either thiophilic or nonthiophilic behavior. These results suggest that thiophilic interaction chromatography is a process that utilizes a previously unrecognized protein–ligand interaction mechanism. We suggest that salt allows the protein into close proximity with the sulfone–thioether group where short-range forces are effective. An electron donor–acceptor or proton-transfer mechanism may be involved. The demonstrated potential for modification of experimental conditions to vary T-gel selectivity makes thiophilic adsorption a powerful new alternative for the noncovalent immobilization and purification of biologically significant macromolecules.

Important molecular (ligand–ligate) interactions can occur entirely in free solution, but in biology these interactions often include interfacial recognition events involving immobilized ligands or acceptor sites which constitute the essence of cellular and subcellular infrastructure. For proteins in an aqueous environment, organized water structure is a characteristic of the protein–solvent interface and may be considered an imporant extension of the protein's molecular surface properties. Indeed, discussions of both the thermodynamic and kinetic limitations of protein structural dynamics (Karplus & McCammon, 1983) as well as the solvent accessibility of individual functional groups (Eisenberg, 1984) are focused (in

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part) on resident water structure. Certainly, the favorable entropy changes associated with the displacement of bound water are an important factor in stabilizing protein–protein and protein–ligand recognition events. The differential ability of various salts to promote as well as destabilize solid-phase protein adsorption illustrates how modifying the structure of water can signficantly influence biologically relevant ligand–ligate interactions. There has recently been a growing awareness of the necessity to more fully understand and better utilize these processes.

High-affinity biomolecular adsorption events, although often useful in vitro, involve chemistries and mechanisms that are typically difficult to ascertain with any degree of certainty. Simple, well-defined model systems are useful to identify and quantify the individual driving forces influencing ligand–ligate interaction selectivity and affinity. Toward this objective, a novel type of protein–ligand recognition process has recently been described (Porath et al., 1985). The precise mechanism of this "thiophilic" interacton process is currently unknown, but interaction of the nonionic sulfone–thioether ligand with appropriate acceptor sites on the protein surface appears to be quite specific, is promoted in the presence of "water-structure-forming" salts, and can be distinguished from generic hydrophobic interactions.

We have recently described conditions whereby a single thiophilic adsorption column can efficiently, selectively, and reverisibly immobilize immunoglobulins from unfractionated human serum under mild conditions (Porath et al., 1985; Hutchens & Porath, 1986). Similarly, bovine immunoglobulins were selectively removed from calf serum utilized for cell culture, and monoclonal antibodies in hybridoma cell culture fluid were purified to apparent (electrophoretic) homogeneity in one step (Hutchens, Ruan, Andersson, and Porath, unpublished results; Porath & Hutchens, 1987). This type of selectivity is unusual and intriguing in terms of mechanism and applicability. It was of interest therefore to determine whether the specificity of the thiophilic adsorption process could be experimentally altered to increase selectivity for other proteins of interest. We report here our investigations of those variables found to alter both the capacity and selectivity of the thiophilic adsorption process.

Materials and Methods

The thiophilic gel (T-gel) used for these investigations was synthesized as described recently (Porath et al., 1985). Spherical beads of agarose (6%) were activated with divinyl sulfone to which mercaptoethanol was attached to a final ligand concentration of approximately 0.9–1.0 mmol (g of dry gel)$^{-1}$. The general structure of this thiophilic adsorbent can be represented as follows: agarose–$OCH_2CH_2SO_2CH_2CH_2SCH_2CH_2OH$. Only previously unutilized T-gel was used for these studies. Chromatographic details are provided in the figure legends. The proteins listed in Figure 1 were all obtained from Sigma Chemical Co., St. Louis, MO, with the exception of normal human serum immunoglobulins (γ globulins) which were generously supplied by KabiVitrum, Stockholm, Sweden, and human carbonic anhydrase I was kindly provided by Professor Sven Lindskog of the Institute of Biochemistry, Umeå University, Umeå, Sweden. All buffers and reagents were of standard quality. The buffer system used for the pH dependence studies (Figure 3) was composed of the following buffer constituents at a concentration of 0.2 M each: citric acid, sodium phosphate, 2-(*N*-morpholino)ethanesulfonic acid, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (Hepes), and glycine adjusted to pH 3–9.

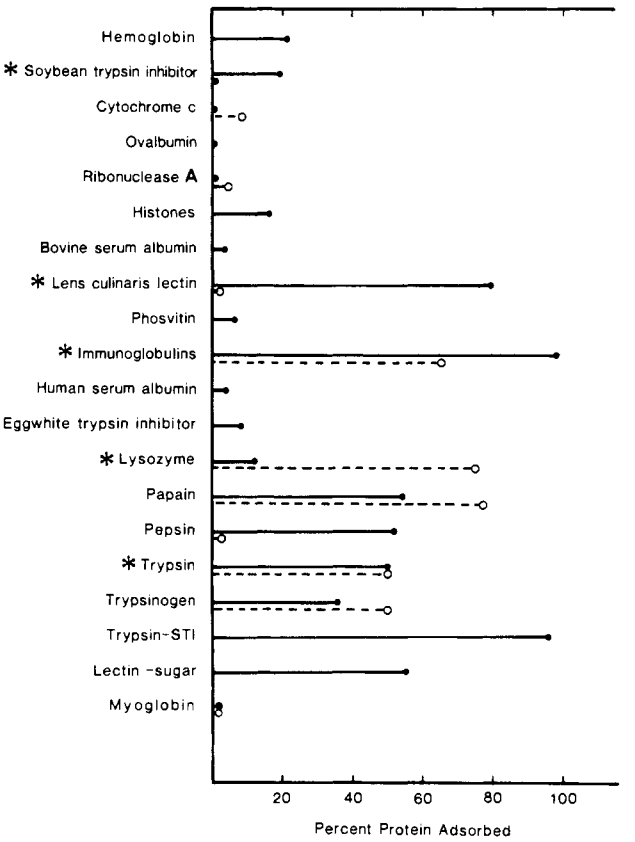

FIGURE 1: Variation in thiophilic adsorption behavior of selected proteins. Chromatography was performed with small-diameter columns (0.5 cm i.d.) packed with 0.205 g of suction-dried thiophilic gel and eluted batchwise (20 °C) at approximately 1 mL/min. Two-milliter aliquots of sample protein (0.5 mg/mL) were applied in column equilibration buffer, which consisted of 20 mM Hepes (pH 7.5 at 20 °C) either with (closed circles) or without (open circles) 10% (w/v) ammonium sulfate. After the column was washed with 4–6 column volumes of column equilibration buffer, protein desorption was achieved by removal of ammonium sulfate and inclusion of 60% (v/v) ethylene glycol. Recovery of adsorbed protein was routinely above 90%. The asterisks denote the five model proteins chosen for further characterization of the T-gel.

Results

Thiophilic Properties of Structurally Diverse Proteins. Figure 1 shows the relative thiophilic adsorption behavior of several proteins varying in size, subunit composition and structural organization, surface charge, relative surface hydrophobicity, disulfide content, and carbohydrate content. The adsorption of these proteins to the thiophilic gel was generally salt pomoted but can be both dependent on and independent of the presence of water-structure-forming salts. Under set conditions, the relative extent of thiophilic behavior revealed by the individual proteins evaluated appeared to vary considerably, and several adsorption patterns were suggested. For example, certain proteins showed little or no thiophilic tendencies in either the presence or absence of water-structure-forming salt (e.g., myoglobin, cytochrome *c*, ovalbumin, carbonic anhydrase I, and ribonuclease A). At the other end of the spectrum, proteins such as the immunoglobulins, papain, and to a lesser magnitude, trypsin were significantly adsorbed regardless of the presence of salt. Additionally, while the thiophilic adsorption of *Lens culinaris* lectin was absolutely dependent upon the presence of water-structure-forming salt, under the conditions specified (i.e., 10% ammonium sulfate) lysozyme was adsorbed to a greater extent in the absence rather than presence of salt.

To further investigate those experimental factors affecting

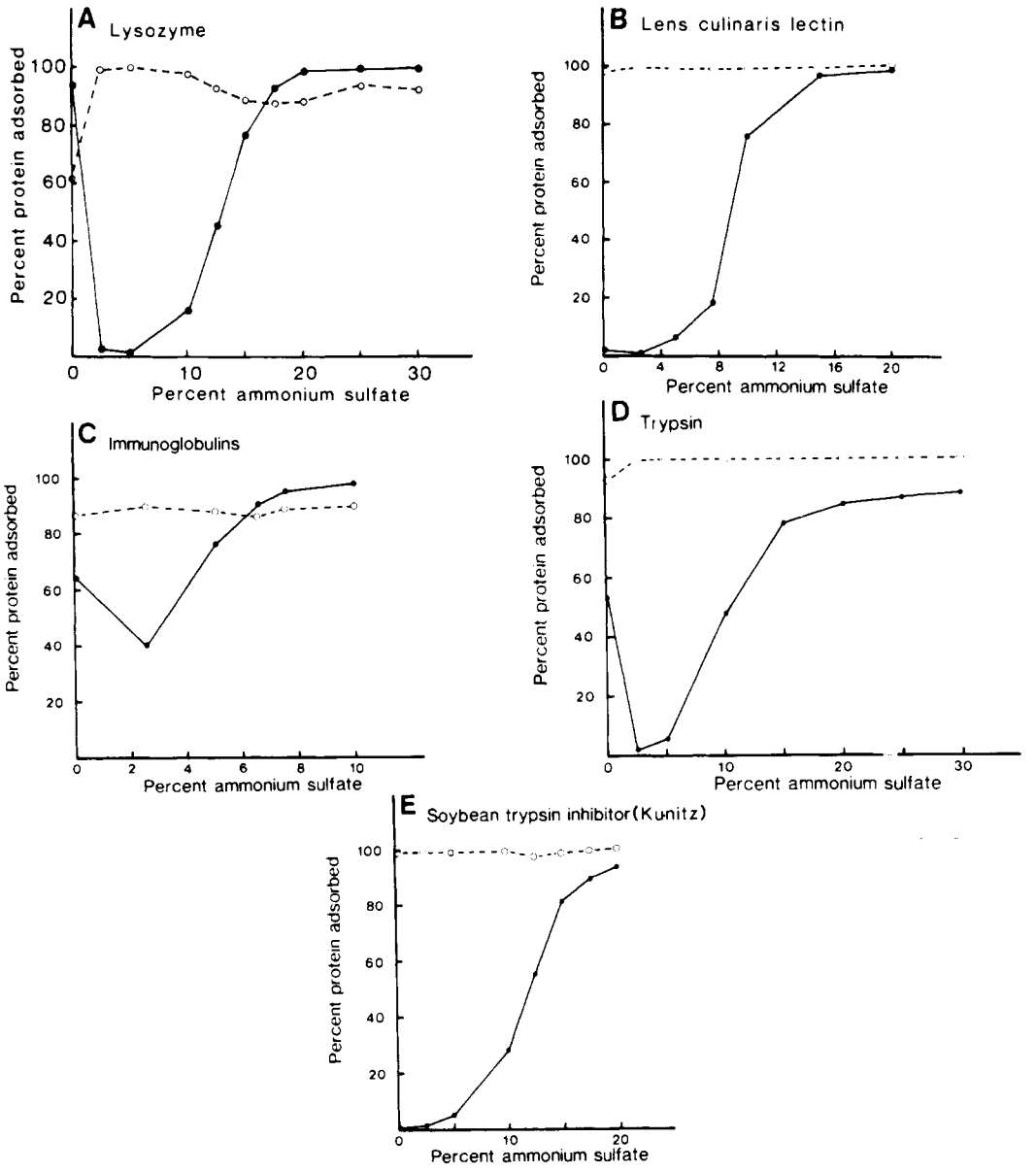

FIGURE 2: Relative dependence of thiophilic adsorption on concentration of water-structure-forming salt (ammonium sulfate). Lysozyme (A), *L. culinaris* lectin (B), immunoglobulins (C), trypsin (D), and soybean trypsin inhibitor (Kunitz) (E) were analyzed for thiophilic adsorption (closed circles) as a function of ammonium sulfate concentration. Adsorption efficiency was determined as described for Figure 1. Protein recovery is indicated by the dashed line (open circles).

thiophilic adsorption selectivity and capacity, five model proteins were chosen. As shown in Figure 1 and as discussed below, these five proteins have diverse physicochemical properties and represent a varied set of affinities for the T-gel in both the presence and absence of water-structure-forming salt.

Variations in Salt-Promoted Adsorption of Thiophilic Proteins. The concentration dependence for ammonium sulfate promotion of thiophilic adsorption is shown in Figure 2 for each of the five model proteins. The *L. culinaris* lectin and soybean trypsin inhibitor (Kunitz) showed no tendency for salt-independent adsorption to the thiophilic gel. The concentration of ammonium sulfate required to promote 50% thiophilic adsorption was 8.5% and 12% for the *L. culinaris* lectin and soybean trypsin inhibitor, respectively. The concentration dependence of salt-promoted adsorption for each protein followed a rather steep sigmoidal curve. In each case, protein recovery was excellent upon desorption with elution buffer containing no ammonium sulfate. In contrast to the results shown for the lectin and soybean trypsin inhibitor, trypsin, lysozyme, and the immunoglobulins revealed different magnitudes of salt-independent adsorption tendencies. However, once a minimum ionic strength was established, the characteristic salt-promoted adsorption curve became evident. For proteins showing any degree of salt-independent adsorption, low concentrations of sodium chloride can be used effectively to prevent adsorption and to achieve nearly quantitative desorption. We have further discovered that the presence of sodium chloride (e.g., 0.1–0.5 M) does not interfere with the ammonium sulfate promoted adsorption process (Hutchens & Porath, 1986).

Influence of pH on Thiophilic Adsorption. A variation of thiophilic adsorption with altered pH is characteristic for each of the five model proteins and is shown in Figure 3. In the presence of ammonium sulfate concentrations normally required to promote equivalent but submaximal (78–91%) adsorption, a distinct adsorption minimum was observed for each protein at or near pH 5. The magnitude of this effect varied with the individual protein. Importantly, when the thiophilic adsorption process is driven to its maximum efficiency at

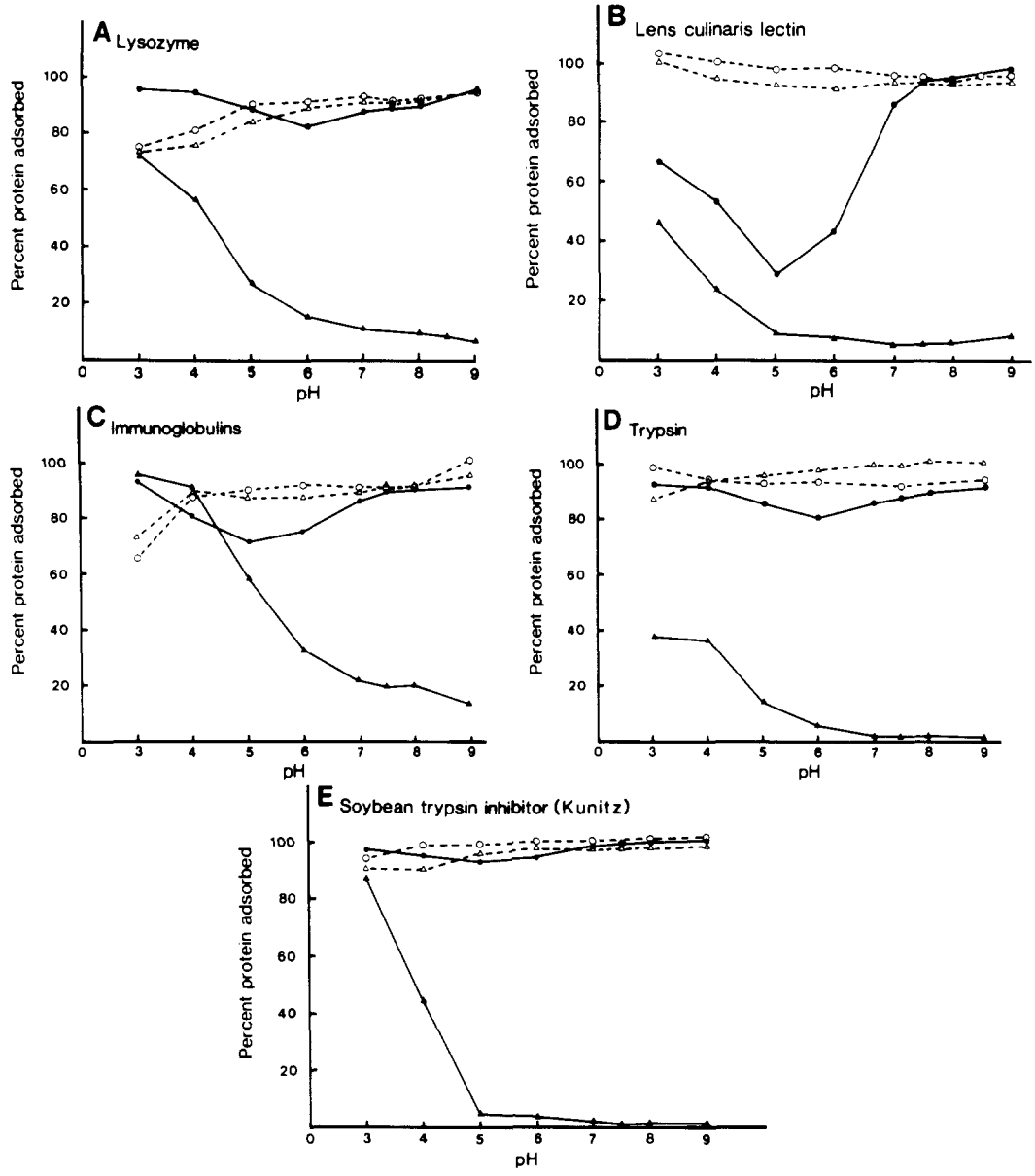

FIGURE 3: Variation in thiophilic adsorption behavior as a function of pH in the presence and absence of water-structure-forming salt (ammonium sulfate). Lysozyme (A), *L. culinaris* lectin (B), immunoglobulins (C), trypsin (D), and soybean trypsin inhibitor (Kunitz) (E) were analyzed for their thiophilic adsorption properties in the absence (closed triangles) and presence (closed circles) of 5% (C), 10% (B), or 15% (A, D, and E) ammonium sulfate. Adsorption efficiencies at each pH were determined as described for Figure 1. Protein recoveries are indicated by the dashed line for proteins adsorped in the absence (open triangles) and presence (open circles) of ammonium sulfate.

higher concentrations of ammonium sulfate, no pH-dependent variations in adsorption capacity were detected. In the absence of ammonium sulfate, each of the five model proteins showed some tendency for salt-independent adsorption at more acidic pH values. Again, the magnitude of this effect varied with each individual protein and was easily reversed. Recovery of the adsorbed proteins was high and consistent regardless of whether the adsorption was salt-dependent or salt-independent (e.g., at acidic pH values).

Variations in Thiophilic Adsorption with Different Types of Water-Structure-Forming Salts. The differential ability of various monovalent and divalent cations of salts from one end of the Hofmeister (1888) series to promote thiophilic adsorption was distinctive for each of the five model proteins as shown in Figure 4. Sodium, potassium, magnesium, and lithium salts of sulfate were each investigated at 0.5, 0.65, and 1.0 M concentrations. The variation in sulfate counterion affects both the concentration of salt at saturation and individual protein solubility (Arakawa & Timasheff, 1984).

In general, sodium sulfate appeared to be the most effective and magnesium sulfate the least effective at promoting the thiophillc adsorption process. However, these differences were not large and varied with the individual model proteins evaluated. In the case of the immunoglobulins and *L. culinaris* lectin, the differences between the various salts were most pronounced at the lower concentrations with little or no differences apparent at 1.0 M concentrations. Soybean trypsin inhibitor and lysozyme were quite varied in their thiophilic adsorption tendencies with the different salts. These results indicate the necessity to consider individual salts (at varying concentrations) for a given separation problem.

Discussion

Thiophilic interaction chromatography may be considered a type of affinity adsorption technique. We have preivously shown the T-gel to be unusually selective and efficient for the immobilization and purification of immunoglobulins from unfractionated human serum or hybridoma cell culture fluid

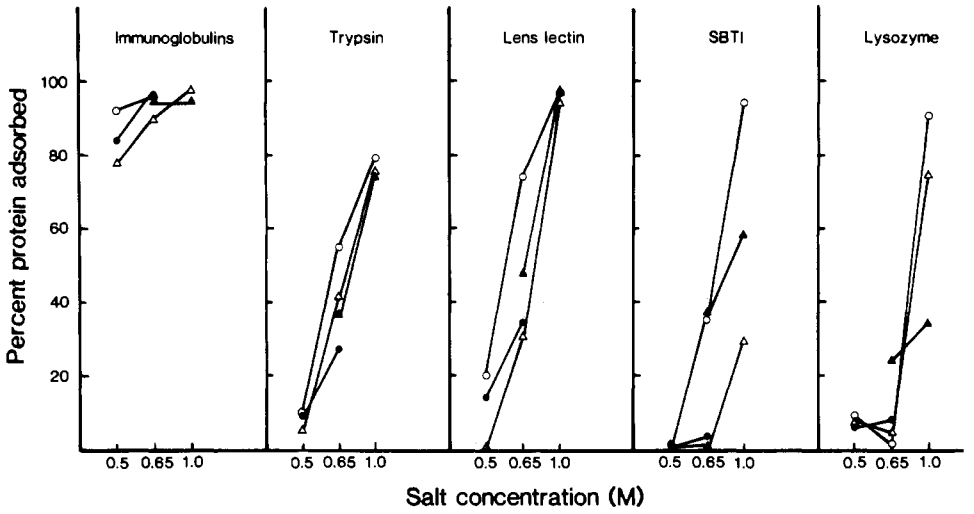

FIGURE 4: Variable promotion of thiophilic adsorption behavior with different water-structure-forming salts. The indicated proteins were analyzed by thiophilic adsorption chromatography in the presence of 0.5, 0.65, and/or 1.0 M sodium sulfate (○), potassium sulfate (●), magnesium sulfate (△), and lithium sulfate (▲). Chromatographic conditions were as described for Figure 1.

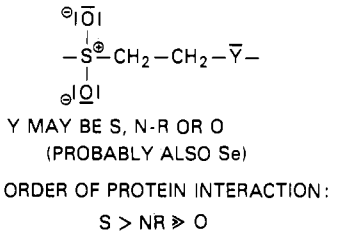

FIGURE 5: Generalized T-gel ligand structure.

(Hutchens & Porath, 1986; Porath & Hutchens, 1987). We have now provided evidence that a variety of other well-characterized proteins can be induced to reveal thiophilic properties. Thus, thiophilic adsorption has potential applications far beyond immunoglobulin purification. The data presented here demonstrate that thiophilic adsorption is a process that can be controlled experimentally by varying one of at least three variables, namely, pH, concentration, and/or type of water-structure-forming salt.

There appears, as yet, to be no overall structural feature that is common among the thiophilic or, conversely, the apparent nonthiophilic proteins investigated. Thus, neither the thiophilic ligand acceptor site(s) on the protein nor the precise adsorption mechanism is known at this time. However, considering the structure of the thiophilic ligand, in this case $-CH_2CH_2SO_2CH_2CH_2SCH_2CH_2OH$ (Figure 5), we can speculate that the permanent sulfone dipole and the free electron pair of the thioether potentially act in concert as electron acceptor and donor sites, respectively. Thus, the 2-carbon proximity of the thioether to the sulfone group may be a requirement for expression of the characteristic T-gel selectivity. Investigations of structurally related immobilized ligands support this suggestion. Still another hydroxyl (–OH) or even a thiol (–SH) can be introduced in the T-gel ligand with characteristic T-gel adsorption capacity essentially retained (Porath et al., 1985):

$$-OCH_2CH_2SO_2CH_2CH_2SCH_2CH(OH)CH_2OH$$

$$-OCH_2CH_2SO_2CH_2CH_2SCH_2CH(SH)CH_2OH$$

Further, the ligand

$$-OCH_2CH_2SO_2CH_2CH_2SCH_2CH(OH)CH(OH)CH_2SH$$

has a similar adsorption capacity (Porath et al., 1985). Thus it seems that thiophilic interaction, from the view of the ligand, should probably be considered hydrophilic rather than hydrophobic. Like the T-gel, the A-gel and DPA-gel (J. Porath, unpublished results) as well as the TCPA-gel (Porath, 1987) shown below selectively adsorb immunoglobulins and α_2-macroglobulin from human serum under the conditions specified. The A-gel is more hydrophilic than hydrophobic,

A-gel agarose-$OCH_2CH_2SO_2CH_2CH_2NH_2$

DPA-gel agarose-$OCH_2CH_2SO_2CH_2CH_2N(CH_2C{\equiv}CH)_2$

TCPA-gel agarose-$OCH_2CH_2SO_2CH_2CH_2NHC(CH_2C{\equiv}N)=C(C{\equiv}N)_2$

and the hydrophobicity of neither the DPA-gel nor the TCPA-gel is strong enough to make it an adsorbent for serum albumin. A ligand with only thioether sulfur, as obtained by coupling mercaptoethanol to oxirane–agarose

$$-CH_2CH(OH)CH_2SCH_2CH_2OH$$

does not convert agarose to a protein adsorbent, and neither will a ligand containing only a sulfone group in an aliphatic surrounding be thiophilic. In fact, the ligand shown below appeared to be relatively inert as protein adsorbent (Porath et al., 1985):

$$-OCH_2CH_2SO_2CH_2CH_2OH$$

Similarly, the following structure (derived from oxirane-activated agarose)

$$-CH_2CH(OH)CH_2OCH_2CH_2OH$$

although similar to the T-gel ligand does not have a sulfone group and was found not to have thiophilic (or any other) protein adsorption property under the conditions evaluated (Porath, 1987). Further, when the tricyanopropenamine (TCPA) group is attached via an oxirane coupler [$-OCH_2CH(OH)CH_2$–(TCPA)] instead of the divinyl sulfone coupler [$-SO_2CH_2CH_2$–(TCPA)], little or no protein adsorption occurs (Porath, 1987). So, it currently appears as though a necessary and probably sufficient structural feature for thiophilic interaction is contained in the sequence $-SO_2CH_2CH_2X-$, where in the absence of aromatic substitutions, X can be any atom with a lone electron pair (S > N ≫ O). The requirement for sulfur in the form of a sulfone group appears to be absolute but not sufficient.

How can this structure so specifically interact with the surface of certain proteins? Related thiophilic adsorption experiments with defined peptides (in progress) strongly

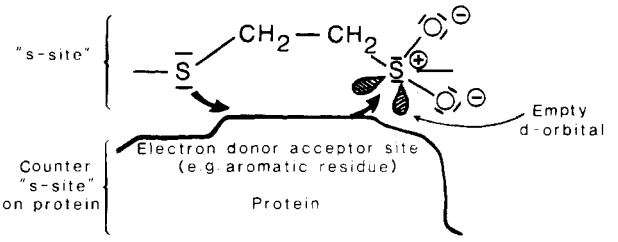

FIGURE 6: Possible interaction mechanism operating during thiophilic adsorption. Of necessity, the interaction must involve the entropy-favored rearrangement of surface-bound water (hydrophobic-like effect) in addition to the proposed electronic coupling.

suggest that aromatic amino acids may be involved. Is the acceptor site a π-electron system such as the indole nucleus of a tryptophan residue located in an accessible cavity or at the surface of the protein molecule? We believe that the sulfone group and thioether sulfur (or other electron donor) cooperate to form some kind of ring structure by transfer of electrons or protons between the ligand site ("S site") and the corresponding countersite on the protein. This electron donor–acceptor relationship is schematically depicted in Figure 6. Other examples of these types of interactions include dipole interactions as well as π-electron sharing events as described for charge transfer (Porath & Larsson, 1978; Porath, 1979). How might the presence of certain salts promote this type of interaction? To the best of our knowledge, the mechanism(s) of salt-promoted adsorption (e.g., to immobilized metal ions, aliphatic hydrocarbons) has (have) not been explained. However in the presence of relatively high concentrations of water-structure-forming salts, protein ligates may differentially accumulate at the phase boundary, having been forced out of bulk solution. This effect may delay diffusion and increase the local concentration enough to alter the equilibrium in favor of enhanced interaction. Understanding the interaction mechanism in detail will require an appreciation of the influence exerted by salt on both water and protein surface structure. Recently, the effects of various cations on protein hydration and salt binding have been evaluated relative to protein solubility and stability (Arakawa & Timasheff, 1984). Work is in progress to determine the precise relationship between salt type, protein hydration, surface tension, and affinity for the T-gel.

Several observations make the classification of thiophilic adsorption as simply a generic hydrophobic interaction process unlikely. Thiophilic adsorption appears to be much more selective than typical hydrophobic interactions. For example, human serum albumin is considered hydrophobic yet is not adsorbed to the T-gel either in the purified state (see Figure 1) or during fractionation of human serum (Porath et al., 1985; Hutchens & Porath, 1986). Similarly, bovine serum albumin, ovalbumin, myoglobin, ribonuclease A, and cytochrome *c* did not interact with the T-gel but are often used as standards to characterize performance of classic hydrophobic gels [e.g., Miller and Karger (1985), Gooding et al. (1986), Hjerten et al. (1986), and Alpert (1986)]. Myoglobin did not interact appreciably with the T-gel even in the presence of 20% ammonium sulfate. Similarly, alcohol dehydrogenases 1 and 2 in crude extracts from *Zymomonas mobilis* were passed unretarded through the T-gel but were adsorbed strongly by both C-4 and C-8 hydrophobic gels under identical conditions (Scopes & Porath, unpublished results). There are also examples of the opposite in selectivity. Immunoglobulins, in contrast to some other serum proteins, are not adsorbed to hydrophobic gels under the same conditions described for maximum adsorption to the T-gel (Porath et al., 1985; Hutchens & Porath, 1986). A separate line of evidence suggesting a class distinction between thiophilic and hydrophobic types of interactions is derived from the disparate salts used to promote adsorption to these column types. Sodium chloride, for instance, is completely ineffective at promoting thiophilic adsorption (unpublished results) but can be used effectively to enhance protein interactions with uncharged amphiphilic (hydrophobic) gels (Porath et al., 1973). Finally, the thiophilic adsorption dependence on pH with a minimum observed at pH 5–6 for each protein investigated appears to be a distinct property of the T-gel but not hydrophobic gels investigated to date.

In addition to the salt-promoted thiophilic interaction property described here, our results indicate the presence of a second, relatively minor, adsorption event which becomes significant at especially low pH values or in the absence of appreciable ionic strength. However, studies with these model proteins suggest that salt-independent interaction with the T-gel at neutral pH may be characteristic of only certain proteins and may not be a general phenomenon. In contrast, the increased adsorption observed at pH 3 has been noted with all proteins examined to date, is salt-independent, and is therefore likely a separate adsorption property of the T-gel or divinyl sulfone cross-linked agarose itself.

In conclusion, we would like to emphasize that the selectivity of the thiophilic gel for many proteins appears to be differently optimized, suggesting a broader spectrum of potential applications than previously recognized.

Registry No. $(NH_4)_2SO_4$, 7783-20-2; Na_2SO_4, 7757-82-6; K_2SO_4, 7778-80-5; $MgSO_4$, 7487-88-9; Li_2SO_4, 10377-48-7; agarose–$OCH_2CH_2SO_2CH_2CH_2SCH_2CH_2OH$, 108423-20-7; soybean trypsin inhibitor, 9078-38-0; cytochrome *c*, 9007-43-6; ribonuclease A, 9001-99-4; trypsin inhibitor, 9035-81-8; lysozyme, 9001-63-2; papain, 9001-73-4; pepsin, 9001-75-6; trypsin, 9002-07-7; trypsinogen, 9002-08-8.

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