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Coprecipitation of Proteins with Matrix Ligands: Scaleable Protein Isolation

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Matrix ligands are agents for isolating proteins out of dilute crndes by copreupitating pmteins. The ligands have a strong anion sulfonate head which initiates binding to proteins having a positive net charge, Z_{H^+} ~ 5–20. Initial binding tightens protein conformation and starts to squeeze water from conformationally motile pmteins. The tails are stackable hydrophobic organic groups, azoaromatic dyes which draw protein–ligand complexes together. Proteins coprecipitate as pests, in the ligand host matrix. In addition to stacking, ligand tails displace water because of their hulk, and lower the average dielectric constant near charged groups, which reinforces the electrostatic component of binding. Matrix ligands protect proteins, scavenge them from dilute crudes (0.01–0.1 per cent protein), and densify coprecipitates. Detergent ions in low concentrations, 10^{-4}–10^{-5} M also somelimes serve as coprecipitating agents, entangling their tails but prohahly not stacking. Divalent metal ions, Zn^{++}, sometimes are useful auxiliary agents. Preparative scaleability from crudes is demonstrated starting from 100–200 g of raw peanuts and raw pineapple to coprecipitate a lectin and bromelain enzyme respectively in 1–2 h with 80–90 per cent activity yields. Ligands are released from coprecipitates by shifting the pH and trapping the ligands with exchange resins. Protein conformation tightening in solution is seen by viscosity measurements.

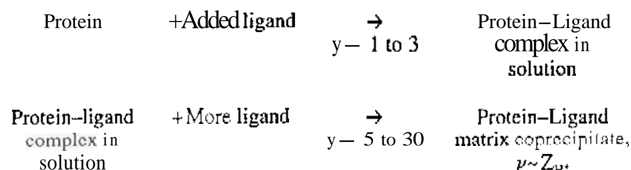
Keywords: proteins; ligands; coprecipitation; precipitation; binding: matrix stacking; isolation; matrix host; protection; forces; scaleability; conformationnl motility

Introduction

A recurring problem in protein isolation and purification is in 'upstream' processing; how to deal with large volumes of dilute crudes. The oldest, most successful general means is to salt out sought-for proteins with kosmotropic salts: ammonium sulfate and sodium sulfate. An advantage of amninnium sulfate is its water solubility, able to reach 4–5 molar concentration. However, two disadvantages derive from large concentrations of inorganic sullates: (a) added steps needed lo get rid of them later; and (b) if very pure salts are not used, cvcn small amounts of heavy metals (ca. 0.001 per cent) in ordinary grades of salt reach levels sufficient to damage sensitive proteins if the proteins are dilute (Scopes, 1993). The goals of the work are to develop matrix ligands for upstream coprecipitation of proteins from crudes, and to take into account protein molecule biophysical behavior in designing such ligands.

Matrix ligands in contrast to conventional salting out, coprecipitate proteins from ligand concentrations in about 10^{-4} M ranges, using ca. 10–20 molecules ligand added/protein molecule in dealing with 0.05–0.30 per cent protein crude solutions. We use the notation, as in previous papers (Conroy and Lovricn, 1992), y=moles ligand added/mole protein when protcin molecular weights are known or can be roughly estimated, and $\nu_{coppt.}$=moles ligand bound/mole protein in matrix ligand–protein coprecipitatcs. The 'co-' prefix is important; protein–matrix ligand complexes mutually coprecipitate one another. A third reactant is much involved in all such interactions and 'hiorecognitive binding', namely water. Water molecules are as critical in the thermodynamics of phase changes in soluble versus insoluble protein equilibria, as the other two components, ligands and proteins. Other authors have emphasized the same point (Lumry, 1995).

Two main reactions outline how matrix ligands do their job. In the simplest form, momentarily leaving out water molecules as the third reactant, they are:



The Z_H+ quantity is the net charge on the protein molecule from the protein molecule's acid–base titration plot. Anion matrix ligands peak in coprecipitation power when there is close to one ligand anion per pmtein cation charge. The overall drive for forming a coprecipitated matrix starts with Coulombic attraction between oppositely charged ligands and protein cationic sites.

Fig. 1 shows the two main reactions in diagram form. The first reaction involves protein molecule tightening of conformationally loose, water-pcnctrated proteins. Proteins in aqueous solution are soluble because they are penetrated by water, to the extent of δ_{H_2O} ~ 0.2–0.6 g H_2O/g protein

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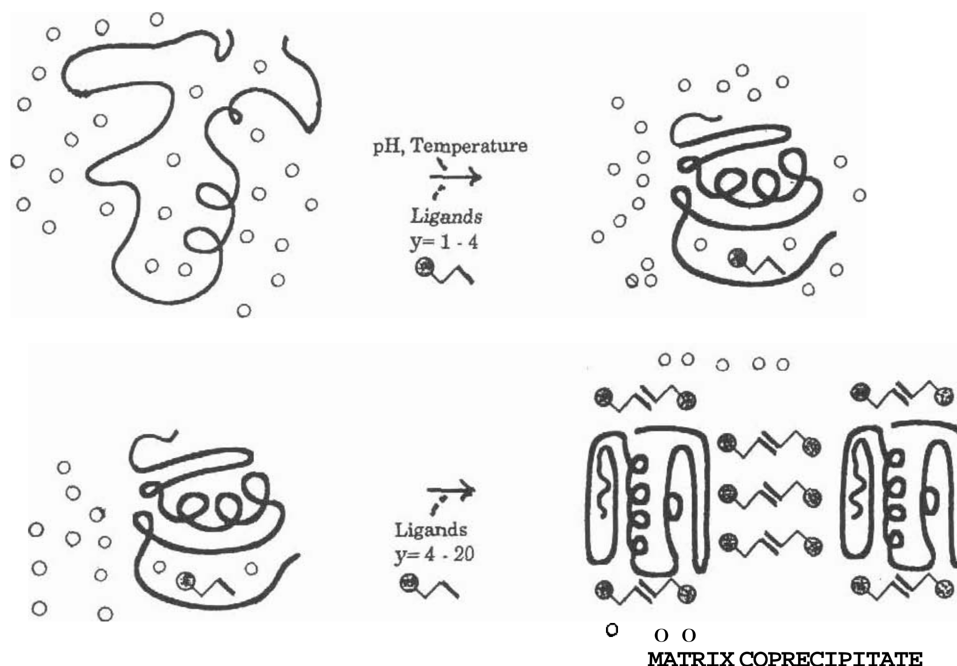


Figure 1. Two principal reactions in matrix ligand precipitation of proteins. The first reaction occurs in solution, initiated by electrostatically driven binding of a ligand to a protein molecule, starting protein conformation tightening (small circles: water molecules). The second reaction is driven by further ligand binding, building the concentration of hydrophobic tails to promote association of complexes, shedding of some water of hydration back to bulk solvent and coprecipitation of ligand–protein complexes. Coprecipitated complexes have a composition, number of ligand anions bound equal to the number of net positive charges on the protein molecule. Quantity y = mol ligand added per nominal mol protein.

(Rupley and Careri, 1991). Conformational tightening can be monitored by several biophysical tools and methods (Lumry, 1995). The second reaction in Fig. 1 produces visible coprecipitation of proteins with their ligands. When protein molecules take on appreciable positive charge, $Z_{H^+} \sim +5$ to +20 in pH ranges about 2–5 pH units below their isoionic point, values quickly reach Z_{H^+} when the overall supply of ligands is sufficient; when y–5–30. Matrix ligands are strongly bound to proteins, shown by a binding isotherm below.

Binding large organic ligands to proteins displaces considerable water, especially if the protein conformation becomes tightened instead of expanded. Bulky organic groups on ligands help lower dielectric constants of the microenvironment around ligand–protein ion pairs, reinforcing binding. Filling available cationic sites on proteins with anionic head groups of matrix ligands, sulfonate groups, brings binding levels to the point where $\nu_{coppt.} \approx Z_{H^+}$. Near or at that point, ligand tail-tail stacking occurs; matrix formation and coprecipitation also occurs. Simultaneously

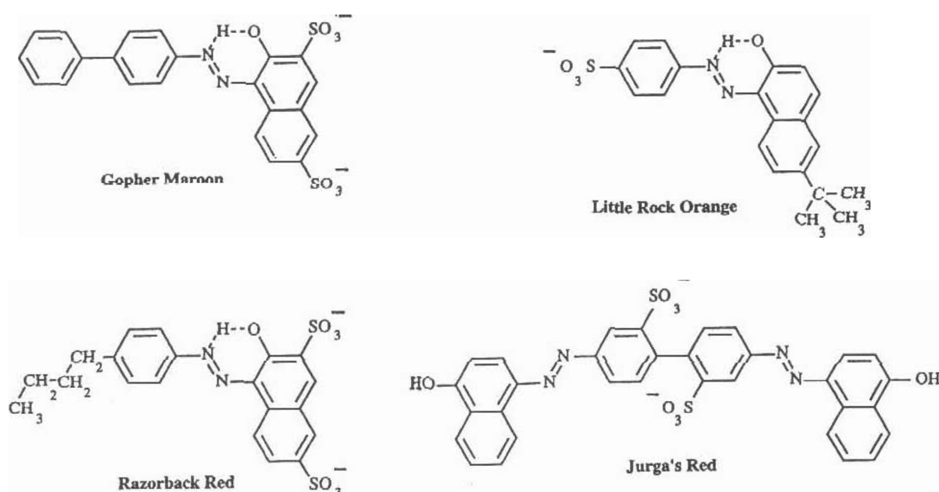


Figure 2. Structure of four azoaromatic sulfonate ligands. Three are single ended, head-tail types, Jurga's red has a mid-section bearing sulfonate groups which generates the electrostatic component of ligand anion binding to protein cation groups.

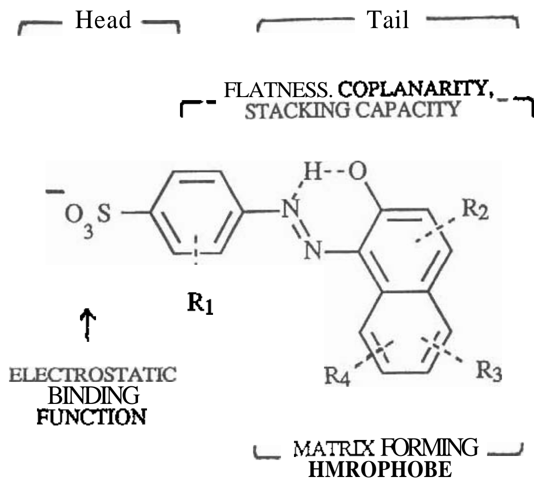


Figure 3. Structure-function contributions to azoaromatic sulfonate lignads for cationic proteins with net charge $Z_{H^+} \sim +5$ to +20:

(i) The SO_3^- head is bound by one protein cationic site in acid pH via electrostatic attraction; the prime determinant in binding stoichiometry expressed by ν_{coppt};
(ii) The azoaromatic structure provides a flat or nearly flat, stackable, hydrophobic tail for matrix building.
(iii) o-hydroxyl azo group tautomerism and internal hydrazone formation reinforces aroaromatic coplanarity;
(iv) R groups=C_3, C, alkane hydrocarbons, add hydrophobic character and bulk water displacement capacity, lowering of local dielectric constant near ionic head groups. Polarizability of ligands adds to their tendency to bind to protein molecules.

proteins become well protected against denaturing forces such as heat and extremes of pH. Ability to use pH extremes is an advantage in work-up and extraction of crudes in some cases, for example when proteases are contaminants.

Matrix ligands tend to facilitate centrifugation of coprecipitates because they are made more dense than precipitated proteins lacking such ligands. There are two reasons. First, water is squeezed out by protein conformation tightening. Normally hydrated proteins tend to be water penetrated, frequently slow to centrifuge or filter; gummy. Water bas a large specific volume $\bar{v} = 1.00$ cm^3/g, relative to all polypeptides with their $\bar{v} \approx 0.72$–0.73 cm^3/g. Therefore water has a markedly lower effective density than the dry peptide. Hence conformation tightening and water squeeze out densifies the protein hydrodynamic particle. Second, matrix ligands have rather small specific volumes, ca. 0.6–0.7 cm^{3}1 g, thus decreasing coprecipitate buoyancies and increasing their effective densities (Conroy and Lovrien, 1992). These same properties are associated with matrix ligand abilities to cocrystallize proteins. They point up the need to regard water, not simply as a solvent for proteins, but as a structural component inside proteins (Baker, 1995) and as a third reactant of as much importance as the other two reactants in unit process engineering, ligands and proteins. Fig. 1 is rather exaggerated in case of some proteins which are already compacl beforehand; before ligands are added. However displacement of even a modest amount of water necessary for keeping some proteins in solution, is frequently enough to produce critical global conformational rearrangements leading to phase changes (Baker, 1995). That is, to precipitation or coprecipitation, and densification of such products.

Three Kinds of Protein Coprecipitating Ligands; their Structure-Function Properties

Three kinds of coprecipitating ligands are used. Four azoaromalic ligands are shown in Fig. 2. They are all strong anions (not ordinarily titratable by H^+ in water) by virtue of their sulfonate groups. Three of them, Razorback red, Gopher maroon and Little Rock orange tend to be coplanar and flat in their organic tails, for reasons indicated in Fig. 3. There arc two intramolecular contributions to coplanarity:

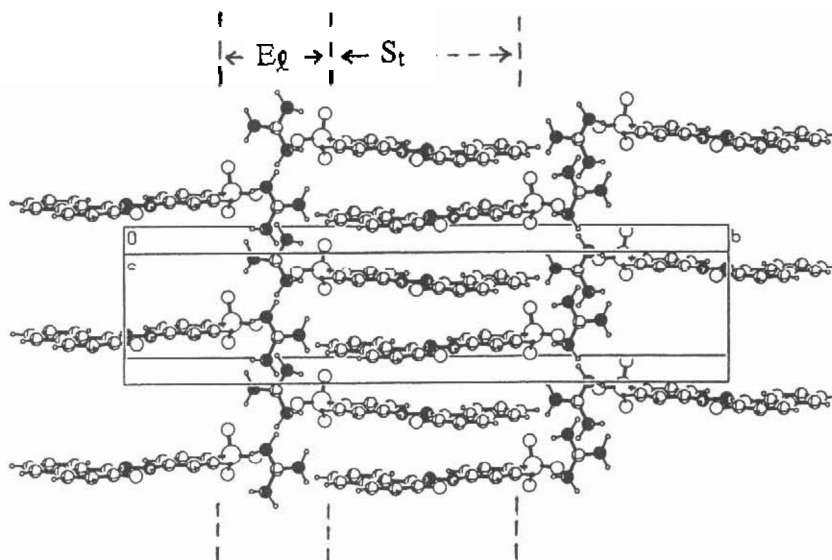


Figure 4. X-ray analysis of matrix ligand Orange G anion cocrystal with guanidinium cation. E_l=electrostatic attraction; S_t=stacking attraction between planar, flat ring organic groups which direct cocrystal packing similar to matrix ligand tail stacking attraction involving proteins (Ojala et al. 1994).

constitutive coplanarity from aromatic ring resonance and conjugation through the diazo linkage. Also, H-atom tautomerism between *ortho* hydroxy groups and their adjacent diazo group markedly favors coplanarity. Evidence for such tautomerism and internal hydrazone formation is reported in several papers based on X-ray crystallography (Olivieri *et al.*, 1989). The *ortho* hydroxy groups in such ligands contribute to hydrophobic (versus hydrophilic) character when the ligand tails stack and draw together proteins to which their heads are bound. Often it is routinely thought that hydroxyl groups are inevitably hydrophilic in character. However there are innumerable instances where, when hydroxyl groups lend strong hydrogen bonding to adjacent groups, well hydroxylated molecules expel and repel water (Odegaard *et al.*, 1984).

Stacking and matrix formation also is reinforced by

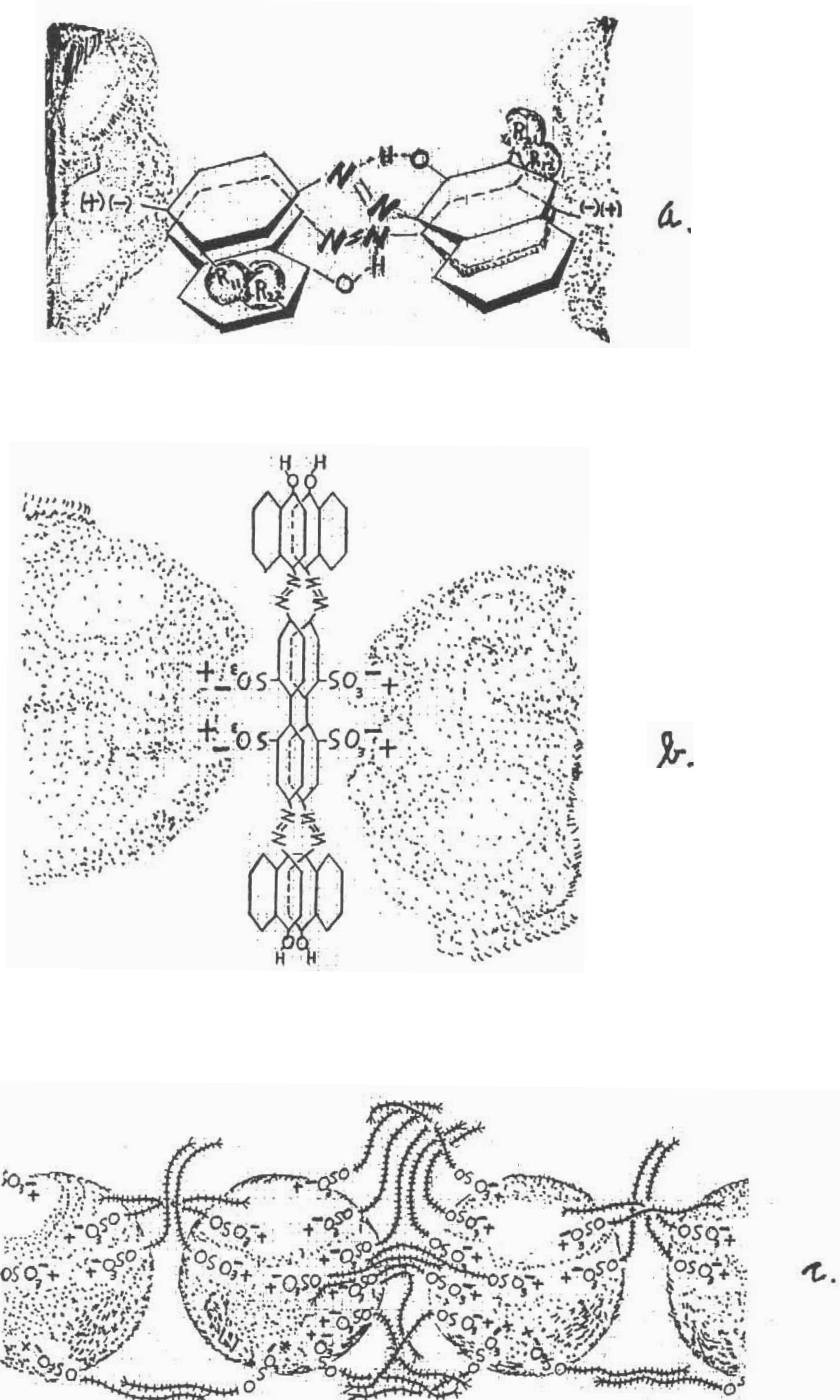
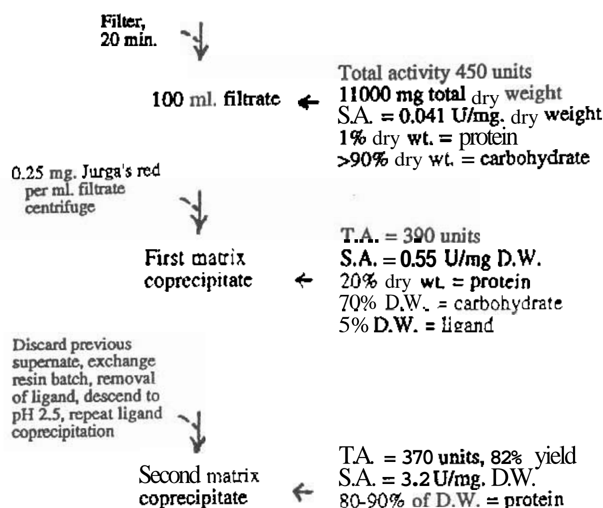


Figure 5. Three means of forming ligand-protein coprecipitates. (a) Matrices formed by stacking and hydrophobic interaction between organic tails. (b) Matrix formation between twin pairs of tails which stack. (c) Detergent anion SDS⁻ flexible tail entanglement (used in overall concentrations far below the cmc)



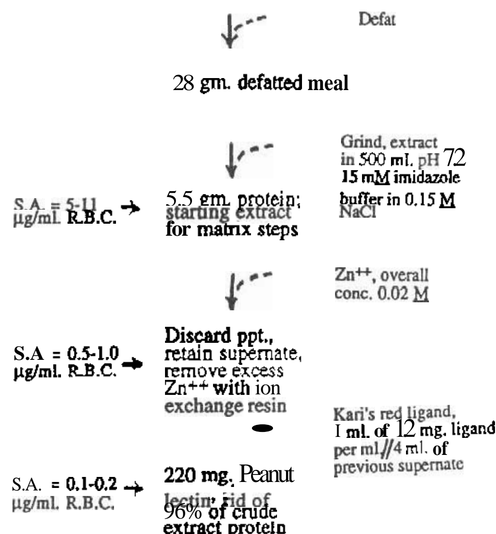


Figure 6. Scale-up of upstream matrix ligand processing raw materials for (a) isolation of bromelain enzyme (pineapple slurry) and (b) lectin (peanut extract). The lectin isolation is benefitted by insertion of a zinc ion step. Kari's red ligand is a t-butyl group substituted naphthalene disulfonate-α-naphthol azo compound. Both these series can be taken through their steps in about 1.5 h or less.

alkane R_1, R_2, R_3, R_4 groups indicated in Fig. 3. Methyl and ethyl alkane groups are fairly effective. However, the four carbon t-butyl and n-butyl groups on two of the ligands shown in Fig. 2, and the additional benzene group on the Gopher maroon ligand add hydrophobicity when ligand–ligand association, stacking and matrix building get started. Some of the ligands shown in Fig. 2 related to such ligands described before (Lovrien *et al.*, 1994) may be synthesized with two sulfonate anions. They maximally coprecipitate proteins at the binding level, $\nu = 1/2\ (Z_{H^+})$ because two sulfonates on each ligand are countered by two protein cationic groups.

Jurga's red ligand efficiently coprecipitates bromelain from crude pineapple slurry. Jurga's red contributes a second kind of matrix ligand. It is 'double ended', having two azoaromatic tails linked to the mid-section biphenyl disulfonate. The dianion mid-section is attracted to bromelain cationic groups in acid pH. Thence the azoaromatic tails stack to form the matrix for coprecipitating the protein molecule. The outboard hydroxyl groups probably do not tautomerize, but possibly are bridged by a water molecule. The ligands appear to 'osculate'; associate pairwise to draw protein molecules together starting from electrostatically initiated ligand binding, similar to the single-ended ligands like Razorback red. Stacking and π face $-\pi$ face association, the basis of matrix formation, is illustrated in Fig. 4 from X-ray crystallographic analysis of Orange *G* anion cocrystalline salts with metal cations (Ojala *et al.*,

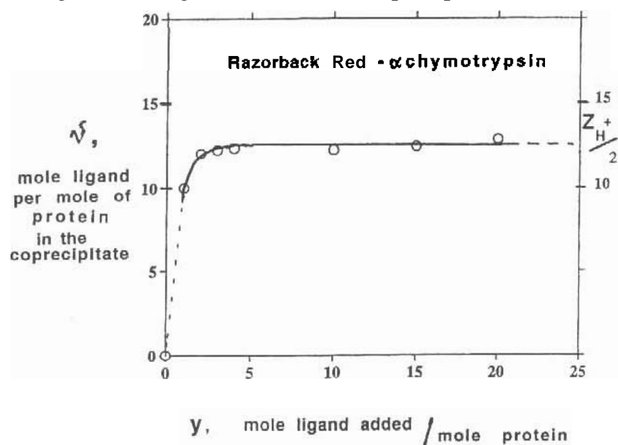


Figure 7. Binding isotherm for a matrix ligand to α-chymotrypsin at pH 3.2, 25°C. Left ordinate, mol ligand bound/mol protein in the coprecipitate. Right ordinate, charge on the protein. Z_{H+} (monovalent ligand) or $Z_{H+}/2$ (divalent ligand). Capacity to reach maximum ν_{coppt}, value (plateau) with small y values ($y \ll \nu$) is strong evidence for 'pulling' reactions to the right in Fig. 1; product (coprecipitate) stabilization. Compare with Fig. 8.

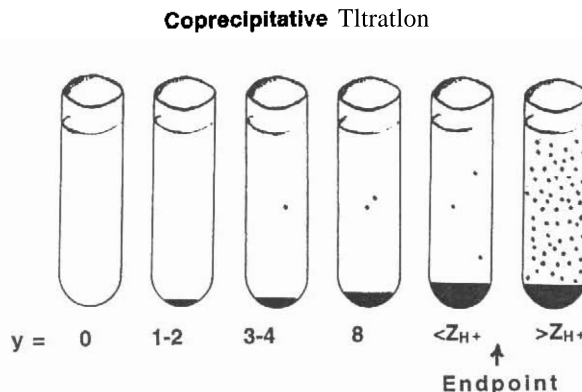


Figure 8. Nearly all ligand added to a protein capable of matrix coprecipitation enters the coprecipitate to give a product of almost constant composition (plateau of Fig. 7), through most of the range of y. Strong coprecipitation during addition of ligand leaves no, or very little ligand in the supernate until the endpoint is reached, bringing down all available protein (coprecipitative titration), after which excess ligand remains in the supernate.

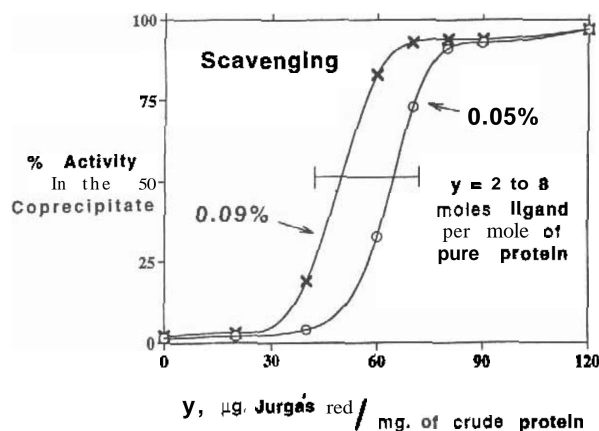


Figure 9. Scavenging of bromelain enzyme from dilute crude protein, 0.05–0.10% with Jurga's red ligand, pH 2.3, 25". Mid-points of plots: are equivalent to y=4–10 mol ligand addedlnominal mol protein (MW~23 000). Coprecipitation time, 1 h max. Enzyme activity recovered from centrifuged coprecipitate by shifting the pH to 5 followed by anion-exchange resin trapping the ligand (Dowex 50-Cl⁻ form).

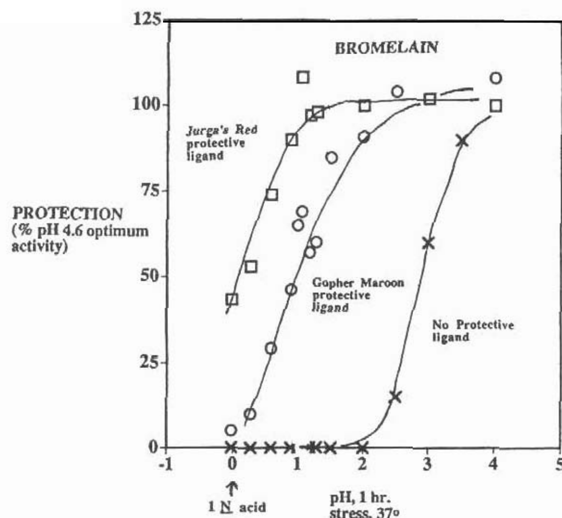


Figure 11. Protection of bromelain enzyme against acid pH, 37°C, 1-h exposure, using two matrix ligands, Gopher maroon and Jurga's red.

1994). When cations are quite small, as in guanidinium and divalent metal cations, cocrystals of this kind are very strong, often rather insoluble in water.

The third kind of ligand shown in Fig. 5 is simply a detergent sulfate; dodecyl sulfate and closely related detergents. Their Rexihlc hydrocarbon tails can strongly entangle and efficiently coprecipitate numbers of proteins. The hydrocarbons also displace water. It is necessary to govern the pH range within rather narrow limits (about ±0.5 pH unit). The system maximizes its coprecipitating powers when there is fairly precisely one SDS–(sodium dodecyl sulfate anion) bound per protein positive charge, Z_{H^+}, as in the case of the azo dyes. The first well organized research concerning detergent anion coprecipitation of six proteins was reported in 1944 by Putnam and Neurath. (There was an earlier paper, by Bull and Neurath in 1937, reporting the same reaction but provided only one example. serum albumin coprecipitation). Detergents in general and SDS in particular is commonly regarded as a protein denaturant and protein-solubilizing agent. It may surprise

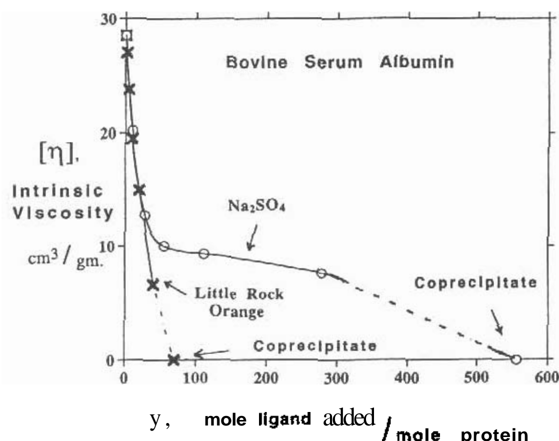


Figure 10. lightening of a protein from acid-expanded conformation shown by its intrinsic viscosity, to a compact form ($[\eta]<5$ cm³/g), by a matrix organic ligand and by simply sulfate anion. Plasma albumin, pH 2, 30 mM in HCl, beginning chloride concentration before ligands were added: 0.03 M.

some that SDS in small concentrations acts as an efficient matrix coprecipitant for proteins, a conformation tightening (versus loosening) ligand, and as a protein protective agent (Lovrien *et al.*, 1994). SDS is also an able protein-cocrystallizing agent for a number of proteins (Lovrien *et al.*, 1994). Distinction between SDS⁻'s opposite kinds of behaviour sharply depends on: (a) the overall concentration of SDS⁻ and related detergents, relative to the critical micelle concentration (the cmc) (b) relative concentration of SDS⁻ with respect to protein concentration, denoted y=mol SDS⁻ added/mol protein. Above the cmc, dodecyl sulfate micelles denature many proteins. It is often said that detergent anions are denaturants but this is inexact. The micelle is the denaturing agent (Tanford, 1980). Well below the cmc, protein–detergent reactions frequently are quite opposite in general nature to their reactions above the cmc. Concentrations of SDS⁻ needed to reach the cmc vary from 1.3×10^{-3} M (at ionic strength 0.50) to 1.6×10^{-3} (ionic strength 0.02) (Tanford, 1980). Taking 0.02 percent protein with a MW~6×10^4 for example, an overall SDS concentration where the cmc forms, roughly $5\times10^{-3}M$ detergent, corresponds to a y value of 1000–2000 SDS per protein. This is a huge ratio, relative to y values needed for matrix ligand coprecipitation and for detergent–protein cocrystallization, or for detergent protection of proteins. Protein cocrystallization with SDS occurs when y_{SDS^-} is small, such that only one to three dodecyl sulfate anions are bound per cocrystallized protein mole as in β lactoglobulin (McMeekin *et al.*, 1949) and lysozyme (Yonath *et al.*, 1977). Coprecipitation (without visible cocrystallization) occurs when ν_{SDS^-} is in the neighborhood of Z_{H^+} for the protein, in pH ranges about 1–4 pH units below the isionic pH. That is, when ν_{SDS^-}, and Z_{H^+} range from about 5–30 depending on the protein.

An imprecise but indicative calculation may be made comparing a rough approximation of surface area that a protein might offer, and the area laid down by detergent alkane groups if they happened to either lie flat, or he confined near a protein's surface as in Fig. 5c. Aggregative coprecipitation appears to occur in a rather narrow range of

pH when protein molecules might be covered. Surface areas of protein molecules (assuming spheres) are roughly $A=4\pi r^2$. We let the radius $r=20$–30 Å for proteins of 20–60 000 MW, thus generating areas from 5000 to 11 000 Å^2. The hydrocarbon's projected planar area along the C_{12} chain length for S D S is about 90 Å^2. We multiply the latter by a factor of X2 because of probable pairwise interactions between alkanes. Quotients between protein surface areas and the alkane effective areas then become roughly 40 ± 20 SDS^- alkane surfaces/protein molecule surface. Such a roughly estimated coverage is in approximate agreement with the ν_{SDS^-} binding parameter and Z_H^+ values from protein titration charge, where nearly complete coprecipilation is consistently observed for the other kinds of ligands in Fig. 5. Again, the overall concentration levels of SDS^- to do this should be and certainly are far below SDS^- concentrations needed for severe denaturation of proteins [ca. 1.4 dodecyl sulfate/g protein (Tanford. 1980)]. Matrix coprecipitation, protein molecule protection and tightening, and protein cocrystallization by detergents such as SDS^-, using adequately low concentrations of detergents, agree with a comment by Tanford (1970), reviewing detergent–protein behavior: 'An important aspect of the problem is that extremely low concentrations of detergent stabilize proteins against all denaturing agents, and that the denaturation induced by detergents . . . is a cooperative process occurring only after the detergent concentration has reached some critical value'. In good parallel to what Tanford said, sulfonate alkane detergents in low relative concentrations behave quite oppositely to that in their large concentration (cmc) behavior in not only precipitation versus solubilization of proteins but also in protection versus denaturation of proteins.

Both kinds of matrix ligands, the rigid 'stacking' kind, azoaromatics, and the flexible alkane hydrocarbon kind, detergents, perform these functions in rather narrow ranges of relative concentrations, ligand/protein, and in somewhat more tolerant ranges of Z_H^+, i.e. of the pH of protein solutions. Within those ranges, they can scavenge proteins sometimes nearly quantitatively even from dilute crudes.

Experimental

The azoaromatic compounds were synthesized by diazo coupling (Saunders and Allen, 1985) of aromatic amine sulionates to phenolic or naphtholic compounds, and coupling aromatic amines with phenol or naphthol sulfonates. Intermediates bearing alkyl groups. R,, R_2 etc. as in Fig. 3, such as t-butyl and n-butyl alkyls had to be synthesized. The t-butyl group was substituted onto naphthols by Friedel–Craft addition of t-butyl chloride to the naphthol, via $AlCl_3$ in pel. ether solvent. In other cases the starting point was an alkyl chloroketone substitution to an aromatic ether in nitrobenzene solvent followed by Wolff–Kishner hydrazinereduction of the ketone to make the alkyl phenol or naphthol. The diazotizable intermediate for Gopher maroon synthesis was 4-aminobiphenyl (Aldrich Co.). After condensation and coupling with the diazonium salts, the azoaromatic sulfonates were crystallized with water–ethanol (dominantly ethanol).

The ligands after synthesis and crystallization have molar absorption coefficients (neutral-acid pH, phenolic groups protonated): Gopher maroon, 23 000 M^{-1} cm^{-1} at 506 nm; Razorback red, 22 800 M^{-1} cm^{-1} at 497 nm: Little Rock orange, 24 000 M^{-1} at 503 nm. Measurement of $\nu_{coppt.}$ was performed by measurement of total protein by bicinchoninic acid analysis (Smith *et al.*, 1985) after removal of the dye ligand via pH shifting and anion-exchange resin trapping the ligand. From the same samples, ligand concentrations were measured using their absorption spectra of redissolved coprecipitate as in reference Conroy and Lovrien, 1992.

Bromelain proteolytic activities were assayed with Hammarsten casein substrate based on Kunitz general proteolytic assay (Kunitz. 1947) activated with L-cysteine (Murachi, 1970) at pH 7.2,. and with CLN carbobenzoxy-lysine-nitrophenyl ester, (Silverstein and Kezdy, 1975). Units quoted in Fig. 6a are caseinolytic (Kunitz) units in total activities, and specific activities (SA) are in Kunitz units/mg protein via dry weight analysis. (1 Kunitz unit=1.0 absorbancy of 280 nm peptides left after trichloroacetic acid or perchloric acid precipitation).

Peanut lectin assays (Fig. *6*) were carried out with human red cells using 2×10^8 red cells/ml in isotonic suspension buffer, using neuraminidase-treated cells to render them reactive to this lectin (Anderson and Lovrien, 1981). The activity unit is the number of micrograms of peanut lectin bearing protein to achieve threshold agglutination, per ml of red cell suspension, at 25°C.

Evaluation of protection for bromelain was done by comparing remaining activity of bromelain enzyme in presence of ligands versus the same bromelain sample lacking ligand, in acidic to severely acid conditions at 37° for 1 h. After the stress conditions, the enzyme sample was returned to pH 4.6 for measurement of activity.

Viscometry of plasma albumin. to illustrate macromolecule tightening, was performed in Cannon–Fenske viscometers, similar to earlier previous viscometry measurements in detergent ligand tightening of plasma albumin (Lovrien, 1963).

Reversal of coprecipitation for all ligands was gotlen by centrifuging down coprecipitates, removing the supernate, then adding a buffer to tiuate the coprecipitate upward to the approximate assay pH. Anion-exchanger resin, Dowex 50 in the Cl^- form in all cases thereupon strips out all dye ligand, and the resin become colored, leaving completely uncolored supernate. About 3–5 fold excess of resin exchange equivalents (ca. 1 milliequivalent per wet ml of resin) should he used. The enzyme, lectin, etc., is released into the solution for further processing or assay.

Results

Matrix ligands stabilize coprecipitates; they pull proteins out of solution

Proteins may he taken out of solution as precipitates by three means. They may be pushed out of solution, pulled out, or a combination of both. The thermodynamic basis of precipitation and coprecipitation is lo raise the chemical

potential of the protein while it is in solution (Timasheff, 1992), lower the chemical potential of the precipitate or coprecipitate (Rothstein, 1994), or try to do both. The water of hydration, structural water in protein molecules is profoundly involved. However the molecular mechanics basis may be easier to visualize. Matrix ligand coprecipitation exclusively pulls proteins out of solution. 'Pushing' is not at all involved, as in salting out or in exclusion-crowding. Matrix ligands act in concentrations 10^4 to 10^5 lower than any pushing agent, or any osmotic stressor can possibly function.

Figs. 7 and 8 illustrate the stability of matrix coprecipitates. Fig. 7 is a binding isotherm plotting the dependence of $\nu_{coppt.}$ versus y ior Razorback red ligand-α chymotrypsin enzyme at pH 3.2. The behavior of $\nu_{coppt.}$, the number of ligands bound per protein molecule ***in the coprecipitate*** where y is small (total of all ligands added per protein molecule in the entire system), only about 2, is already plateauing at $\nu_{coppt.} \approx 12$ or 13. Razorback red bears two sulfonates, so 12 ligands contribute 24 negative charges. The Z_{H^+} on α-chymotrypsin at pH 3.2, is $+24 \pm 2$ (Marini and Wunsch, 1963). There is a rather powerful pull provided by the stability of the coprecipitate to load coprecipitating molecules with a maximum number of ligands determined by Z_{H^+} (or $Z_{H^+}/2$ for disulfonate anions). When y is small, only a small amount of α-chymotrypsin is coprecipitated. But α-chymotrypsin which is in fact coprecipitated already has the full complement of Razorback red ligands on each protein molecule. As more ligand is added, as y is increased. additional coprecipitate forms with the same composition leaving essentially no ligand in the supernate (Fig. 8). Supernates remain nearly completely uncolored, although these matrix dyes have intense absorption and are deeply colored. Not until the endpoint is reached and all enzyme becomes coprecipitated does ligaud remain in the supernate and become clearly, vividly seen. The precipitative titration processes in Figs 7 and 8 are analogous to precipitative titration of Cl^- with Ag^+ to form AgCl which has constant composition and a very small solubility product. They are also much like an antigen–antibody mutual coprecipitation ('immunoprecipitin' titer technique). Only when the component being precipitated out is completely consumed, titratcd to the coprecipitative endpoint, does the next increment of compound used to coprecipitate it out, silver ion in the first example, antigen in the second, matrix ligand in the third, remain in the supcmate to be measured.

The coprecipitating power of matrix ligands enables them to **scavenge enzymes** and **other classes** of protein out of dilute solutions. Fig. 9 shows bromelain enzyme coprecipitation with Jurga's red from 0.05 to 0.3 per cent pineapple slurry crude. Nearly all bromelain activity is retrieved (after trapping out Jurga's red with anion-exchange resin), using y values of ca. 2 to 4 matrix ligands added per mole of protein (assuming the MW of most of the crude protein equals that of bromelain). Similar results, coprccipitative scavenging of pepsin from 0.03 to 0.05 per cent solution with rivanol, ethoxyacridinium cation ligaud (pepsin is an anion) were shown earlier (Conroy and Lovrien, 1992). Bromelain and pepsin both were coprecipitated out with simply SDS in narrow ranges of $y = SDS^-/protein$ and of pH, in the manner indicated in Fig. 5c. The results with flexible ligands will be reported in more detail in another paper.

Divalent metal ion assistance for matrix ligands

Divalent metal ions are sometimes of considerable help in organic anion matrix coprecipitation. One expects bound metal ions should increase protein total charge in addition to the H^+ titration charge, thereby reinforcing electrostatic attraction for anionic ligands. They also tend to tighten protein molecules through using their side chains to chelate, similar to metal-ion drawing of other chelate and macrocycle ligands inwards (Burgess, 1988). Zinc ion, $Zn_{ac.}^{++}$ which is actually $Zn\,[H_2O]_n^{2+} \cdot mH_2O$, $(n+m \approx 6)$ (Bock *et al.*, 1995) has some additional advantages. It is not nearly so pro-oxidative nor so damaging towards sulfhydryl proteins as adjacent transition metal ions, e.g. Cr^{++}, Ni^{++}, Cu''. Fe^{II}–Fe^{III}. Zinc ion has some relatively easily displaced coordinate waters (Bock *et al.*, 1995). Hence there does nor appear to he a severe energy penalty for displacing enough water molecules from the zinc ion allowing Zn^{++} coordination with carboxylate, amine, or possibly sulfhydryl groups on proteins. Used in this way, zinc ions are removed later in the preparation by cation resin (Na^+ form) exchange. Another advantage of Zn^{++} and other divalent ions that may bind to precipitated proteins is their small $\bar{v}$ (partial specific volume). Zinc ions $\bar{v}$ parameter is ***negative*** in sign (Millero, 1972), so Zn'' densifies coprecipitates if many are bound, aiding centrifugation. Zinc ion assistance in coprecipitation is especially helpful for isolation of peanut lectin.

Scaled-up processing of a peanut lectin crude and a protease crude

Figs. 6a and 6b respectively show preparative flow diagrams for scaled-up bromelain preparation from raw pineapple using Jurga's red ligand in a 1 h preparation, and peanut lectin preparation from raw peanuts in a 90 min series of two steps after grinding and extraction of the peanut meal. The peanut lectin preparation uses the Zn^{++} addition technique in tandem with Kari's red ligand to capture highly active lectin in excellent yield, equal in SA to most column chromatographic techniques which are much more lengthy. Bromelain preparations received from the Sigma Co. (a semicrude, 20–40 per cent protein) and from the E.Y. Company (claimed to be crystalline bromelain), yielded SA values of 0.9 and 1.8 caseinolytic units/mg. Hence the SA=3.2 units/mg cited in Fig. 6 gotten from Jurga's red coprecipitation indicates efficient yields and retention of proteolytic activity. considering these steps pertain to upstream crudes in a fairly large scale.

In bromelain preparations from the crude, Fig. 6 shows that much of the dry weight of the most crude filtrate is carbohydrate as expected from a fruit. One of the functions of matrix ligands in cases of this sort is to quickly free proteins from carbohydrate (polysaccharides) besides from unwanted proteins. In any case, the second coprecipitation step produces good overall activity yields, about 82 per cent, and large increases in bromelain SA as shown.

A third enzyme, horseradish peroxidase (HRP) may be cited. Details for HRP, and numbers of other enzyme crudes will be reported in more detail elsewhere. But we now report Little Rock orange [structure shown in Lovrien *er al.* (1994) and Fig. 2], which coprecipitated out many proteins

from peroxidase crude, about ten of them, all unwanted proteins seen in SDS–PAGE gels. In a one-step coprecipitation using y=ten ligands per nominal mole of total protein, in ca. 15–20 min, a coprecipitate is obtained with myriad bands (impurities). In contrast, the supernate contained nearly all the peroxidase enzyme with an RZ (Reinheitzahl) of 3.0. a single band in SDS–PAGE gel under heavy loading conditions, exhibiting the high, sharp Soret absorption band of peroxidase. Thus three enzyme crudes, and a number of other enzymes including proteases and cellulase enzymes, quickly respond to these ligands when pH and y values are optimized. They matrix coprecipitate from crudes in good yields, usually in less than an hour. The scale is limited mainly by availability of ligands for reaching ca. 10^{-4} M ligand concentrations for 0.05 to 1 per cent crudes.

Protection of enzymes by matrix ligands and protein molecule tightening

As Fig. 1 shows, binding of such ligands is believed on several grounds (Lovrien *et al.*, 1994) lo tighten protein molecules from a state of partial expansion and water penetration. When conditions are adjusted to considerably expand and loosen protein conformation, hydrodynamic tools become able to monitor that, and follow subsequent 'tightening reactions'. Measurements of intrinsic viscosities provide rather straightforward means of monitoring macromolecule expansion–contraction, and has been carried out for several organic ion ligands (Lovrien *et al.*, 1994). A problem with the need to make many viscosity measurements is large requirements for protein, a whole gram or even more if several intrinsic visocities, $[\eta]$, are to be measured. However this is practical to do for plasma albumin, for which considerable information about expansion–contraction and water penetration is available (Tanford *et al.*, 1955). Fig. 10 shows intrinsic viscosities for plasma albumin at a pH where it is normally well expanded, together with viscosities dependent on Little Rock orange ligand addition, and dependent on simply sodium sulfate addition. The matrix anion clearly shrinks the expanded protein by large amounts, back to $[\eta] - 5\ cm^3/g$, not far from its well known minimal $[\eta]$ for nearly all such compact globular proteins (Tanford, 1968), ca. 3.5–4 cm^3/g (exclusive of long elliposoids, rods). Upon high levels of organic ligand addition, over 50 mol ligand/mol protein. the protein simply coprecipitates, quite expected for many such proteins. Short of coprecipitation however, major tightening of the protein while in solution is manifest.

Equally interesting, inorganic sulfate does the same thing, up to a point. It decisively shrinks the expanded protein back to $[\eta]$ values of ca. 8–10 cm^3/g, in y levels of 50–300 sulfate ions/protein. It is unlikely they are all bound, so many of the sulfate ions left free may be providing a screening (ionic strength) effect. Nevertheless, their behavior falls in well with similar effects observed by other workers discussed later, regarding protein molecule conformation tightening by inorganic counterions especially bound sulfate. When sulfate levels become very large, y>a few hundred, the albumin molecule simply coprecipitates, indicated in Fig. 10.

Such conformation-tightening reactions fall in parallel with protection experiment results. In work with many enzymes, we have observed significant protection of enzymes stressed in various ways, when mauix ligands are added in submillimolar concentrations. Fig. 11 shows an example in the case of bromelain, and two ligands, Gopher maroon and Jurga's red. Bromelain is rather sensitive to low pH (below pH 3.5) denaturation. But it emerges fully active from pHs well below 2, even from acidities approaching 0.5–1 N (HCl), when protected with matrix ligands, when the pH was returned to normal assay conditions. Probably the enzyme is inactive during the time while it has such ligands bound to it. However once the matrix ligands are removed, it regains the activity it had before the stress cycle. Both chemical stress (e.g. Fenton reagent, oxidative free radicals) and thermal or high temperatures may be protected against using matrix ligands, in agreement with previous papers and reviews (Lovrien *et al.*, 1994).

Discussion

Compounds such as the matrix-coprecipitating ligands in Fig. 2 may first appear to derive their function from their organic 'tails': the flat stackable kind, and the flexible hydrocarbon detergent kind led by dodecyl sulfate. Precipitation of proteins by salts, largely ammonium sulfate known as a kosmotrope, so well reviewed by Collins and Washabaugh (1985) may first seem based on quite different principles. Sulfate ion in large concentrations, 0.5–4 M, pushes proteins out of solution whereas matrix ligands of the kind shown in Fig. 2, in 10^5 times lower concentrations pull proteins out by profound stabilization of the product (Fig. 11). Organic matrix ligands pull using attractive forces between ligand tails.

Ligands of the kind shown in Fig. 2 may appear to derive their coprecipitating powers from their organic tails; both the flat, rigid stackable azoaromatic tail, and the flexible alkane detergent kind of tail which entangle with one another. Both these organic anions are normally used in low, carefully metered overall concentrations of 10^{-4}–10^{-5} M. In contrast, precipitation of proteins simply by inorganic sulfate ion. SO,' the leading kosmotrope (Collins and Washabaugh, 1985), normally used in 0.5–5 M concentration, may seem a little different sort of business, based on very different principles. Inorganic sulfate in 0.5–5 M concentrations pushes proteins out of solution. Organic matrix ligand anions of the kind shown in Fig. 2 in 10^{-4}–10^{-5} M concentrations pull proteins out of solution as indicated in Fig. 1, clearly shown by their binding isotherms as in Fig. 7.

At first sight, these two means of precipitating and coprecipitating proteins seem remote, quite unlike one another. Yet they have some important sectors of overlap, considerable common basis. A few sulfate ions from inorganic sulfate when it is used with proteins are rather often seen to bind to protein molecules, via electrostatic forces. Bound sulfate ions help tighten protein conformation (in positively charged proteins) and help squeeze out some

water of hydration. Only a small portion of such sulfates, probably less than I per cent of added sulfate in conventional salting out or in protein crystallization, are so engaged. It largely occurs when proteins have either a positive global charge Z_H+ or smaller constellations of net positive charge. In practice, inorganic sulfate and organic ligand sulfonates are both able protective agents, besides coprecipitating agents for proteins. The structural feature carried by the organic anion sulfates and sulfonates, in great contrast to simply inorganic sulfate, is, of course, the large organic 'tail' born on the matrix ligands. The facets of common behavior, showing how inorganic sulfate behaves in a muted, yet rather generally occurring way in parallel to the organic sulfates is well illustrated by two recent papers by Fink *et al.* (1993) and Goto *et al.* (1990). and in a review by Chakrabarti (1993).

The background of the Fink *et al.* work is that severe acid unfolding of proteins was traditionally interpreted as conformation expansion, profound water penetration, driven hy intramolecular electrostatic repulsion (Tanford, 1968). It is supported by hydrodynamic measurements — of several kinds — or numbers of proteins. However, in 1990 a major change in direction occurred based on three proteins studied by Fink *et al.* (Groto *et al.*, 1990). They forced maximal conformation expansion by acid, pH 3–3.5 on their proteius. Upon even further forcement with severe acid conditions however, reaching pH 1.5–2, the three proteins all contracted, monitored by two spectroscopic and one hydrodynamic tool. This work was repeated 2 years later with other proteins, e.g. staphylococcal nuclease (Fink *et al.*, 1993). Sometimes even chloride (not very polarizable) induced tightening, detected by fluorescence and Fourier transform infrared (I.R.) spectroscopy. Capacity to tighten conformation of acid-expanded proteins correlated with anion size and polarizability: percholate, trichloroacetate and ferricyanide are most effective. In a sense some of these anions can be too effective because sometimes they induce precipitation on the way to recompaction and tightening the expanded molecules in solution. An important conclusion by Fink *et al.* is that these inorganic anion effects are not simply screening (ionic strength) effects. They depend on anion binding. Such binding is promoted by the large cationic charge Z_H+ on the proteins in low pH. The Z_H+ charge is partly neutralized by bound anions. Summarizing so far, inorganic anion binding, its consequences for well expanded protein molecules in tightening them with electrostatic repulsion was the basis for expansion, is exemplified by Fig. 10. Those results align well with the work of Fink *et al.* who used other biophysical tools.

The Chakrabarti review (1993) reports anion-binding sites for $SO_4^=$ and phosphate for **34** protein structures in X-ray crystallographic analysis. The Chakrabarti review shows that ionic sulfate and phosphate, normally acting as kosmotrnpes to push proteins and other solutes out of water, promote an additional function in sub-kosmotropic ranges of salt concentration. Sulfate and phosphate anions rather commonly bind to discrete positively charged sites, act as cocrystallizing agents (a point also raised by Conroy and Lovrien, 1992). Hence these few salt ions in contrast to most such salt ions in the trapped solvent (freely swimming interstitial salt ions) generate a regular periodic X-ray scattering pattern.

On the way to salting out proteins and crystallization of proteins as commonly viewed starting from large kosmotropic salt concentrations, the early protein site binding reactions of sulfate and phosphate, which can occur in lower concentrations, tend to be drowned out. However, their consequences show up later as discrete site binding — for a few of such salt anions — in X-ray analysis. Chakrabarti indicates such discrete site bound sulfates and phosphates frequently confer stability to folded, ordered proteins. These four properties — conformation tightening and water squeeze-out, protein molecule protection, conformation ordering of a kind which may be prerequisite for crystallizability (cocrystallizability) and precipitation — are of one piece. All four can be produced by what happens to conformationally loose, well hydrated protein molecules in solution, wrought by the relatively few inorganic anions that are likely binding to discrete sites, and wrought by organic anions that certainly are binding, probably into some of the same sites. The dissimilarity between inorganic sulfate and matrix ligand sulfates is seen in the requirement that, after binding a few inorganic sulfate ions, many more must be added to get inorganic sulfate to exert its kosmotropy in the 0.5–5 M concentration. Organic matrix ligands merely need to become hound into probably the same few sites, five–20 of them, and that is all. Their 'tails' do the rest of the work of precipitating (coprecipilating), thus requiring only 10^{-4}–10^{-5} M overall concentrations. The signal that both kinds of ligands key their initial binding reactions to electrostatic forces (simple Coulombic forces) is the requirement to use pH conditions acid enough to vest proteins with a positive charge, roughly ranging from $Z_H+=+5$ to $+20$. The critical charges are not simply the global or average charge, hut charges on sites where anion binding makes an impact on conformation and water squeeze-out.

Organic matrix ligands, sulfonate azoaromatics and sulfate detergents amplify what inorganic sulfate alone can either already do or nearly do. However the organic matrix ligands add considerably more force, free energy drop to the summed reactions of Fig. 1. Only a relatively few matrix ligands are needed to coprecipitate proteins. They decisively switch coprecipitation to exclusively a 'pulling' process.

Specificity is not easily predicted. It depends on how the three dimensional structure of protein–ligand complexes in solution arrange themselves in the aggregate, how well or poorly the complexes squeeze out water to associate themselves. The next work should he to synthesize ligands carrying biochemically specific groups, antigens, amino acids, or nucleotides targeted for individual proteins. It may not be necessary to use only azoaromatic, or detergent alkane tails. However they are good choices now and provide much scope.

The work with matrix ligand ions, and inorganic salt ions. has hearing on chromatographic techniques which involve some of the same components. They predict that some chromatographic techniques could be aided by co-injecting agents, even small amounts of detergent anions, to tighten the conformation of protein molecules during chromatography, perhaps at elevated temperatures. Of course, the aims of chromatography mostly are unlike those of matrix ligand coprecipitation. Chromatography is mainly a downstream

technique for relatively small samples, requiring keeping proteins in solution. Matrix ligand technique, 'precipitation', is intended for upstream large-scale use, bringing proteins out of solution.

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