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The Role of Protein Structure in Chromatographic Behavior

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Chromatographic retention is determined by a relatively small number of amino acids located in a chromatographic contact region on the surface of a polypeptide. This region is determined by the mode of separation and the amino acid distribution within the polypeptide. The contact area may be as small as a few hundred square angstroms in bioaffinity chromatography. In contrast, the contact region in ion exchange, reversed phase, hydrophobic interaction and the other nonbioaffinity separation modes is much broader, ranging from one side to the whole external surface of a polypeptide. Furthermore, structural changes that alter the chromatographic contact region will alter chromatographic properties. Thus, although immunosorbents can be very useful in purifying proteins of similar primary structure, they will be ineffective in discriminating between small, random variations within a structure. Nonbioaffinity columns complement affinity columns in probing a much larger portion of solute surface and being able to discriminate between protein variants.

INTERFACIAL PHENOMENA AND SURFACE-SURFACE INTERACtions play a key role in the organization and control of biological systems. Gene duplication, enzyme catalysis, hormonal regulation, membrane synthesis, and the organization of multiple enzyme complexes all depend on surface recognition. These highly specific interactions occur only when there is sufficient geometric complementarity between enough groups on the surface of two macromolecules to initiate intermolecular docking. Although docking is driven by weak forces such as hydrogen bonding, electrostatic attraction, and hydrophobic interactions, the total force of interaction is very strong because of the involvement of multiple groups.

In this article, I will show that there is considerable similarity between the variables that direct intermolecular docking in biological macromolecules and those that determine the chromatographic behavior of proteins. On the basis of current knowledge of protein-protein interactions and the fact that proteins are three-dimensional matrices with great diversity in the distribution of surface functional groups, one would predict that (i) it is sterically impossible for all of the amino acid residues to come in contact with the surface of a chromatographic sorbent simultaneously, (ii) only those residues at or near the exterior surface of the protein will have a major impact on chromatographic behavior, (iii) only a fraction of the external amino acid residues interact with a particular type of chromatography matrix, such as an ion-exchange sorbent or bioaffinity matrix, (iv) heterogeneity in the amino acid distribution at the protein surface may allow portions of the external surface to dominate chromatographic behavior, (v) the region of the external surface that determines chromatographic behavior may vary between chromatographic modes, (vi) structural changes in a protein that alter amino acid composition in the solute-sorbent contact region will alter chromatographic behavior, and (vii) the chromatographic matrix could alter the structure of an adsorbed protein. Because the systematic study of variables that control chromatographic behavior of proteins is still in the early stages, these hypotheses can only be considered a preliminary working model. Evidence supporting this model and the implications of the model will be presented below.

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Steric Effects

Although logic alone suggests that steric phenomena play a role in the interaction of large molecules with a surface, it is possible to show quantitatively that this is true. More than 150 years ago, Wray and Roy (*1*) observed that ions adsorbed to clays could be exchanged stoichiometrically with ions in solution. On the basis of this observation, a quantitative treatment of retention in ion-exchange chromatography (IEC) has been developed (*2*). In this treatment it is assumed that weakly adsorbed ions (D_b) are displaced from ion exchanging ligands on a chromatographic sorbent by charged groups on a protein (P_o).

$$P_o + Z \cdot D_b \rightleftarrows P_b + Z \cdot D_o$$

The subscripts o and b refer to the free and bound forms of ions, respectively, and Z designates the stoichiometry of the process. In the case of polyelectrolytes, chromatographic retention (k') is related to the number of charges (Z) that interact with a sorbent matrix and the concentration of the displacing agent ($[D_o]$) by the equation $k' = I/[D_o]^Z$. The constant I is composed of a cluster of

constants related to the equilibrium constant for the process, stationary phase ligand density, and the stationary to mobile phase ratio. By using this equation, it is possible to determine the Z number of a solute through the relationship of k' to $[D_o]$.

In anion-exchange chromatography of oligonucleotides with less than eight residues, Z was shown to have physical meaning and is equal to the number of negative charges in the molecule (*2*). However, if there are more than ten residues in an oligonucleotide, Z falls below the number of charges in the molecule. This is attributed to the initiation of base-stacking in larger oligonucleotides, sterically preventing all the phosphodiester residues from simultaneously contacting the surface of the chromatographic sorbent. This hypothesis was confirmed by using the highly structured phenylalanyl–transfer RNA (tRNA) (*2*). Z was less than 25% of that expected from the number of charges in the molecule. Partial denaturation of the tRNA with organic solvents increased Z as expected.

Similar observations have been made with proteins (*3*). The stoichiometric displacement model indicates that fewer than ten charges acting simultaneously determine the chromatographic behavior of proteins in IEC.

The displacement model of retention has also been applied to reversed-phase chromatography (RPC) (*4*). Because octyl or octadecyl residues are only 7 Å apart on the average RPC sorbent, reversed-phase media are very hydrophobic and adsorb proteins very strongly. These strong hydrophobic interactions and the presence of underivatized silanol groups on the RPC sorbent make it necessary to use organic solvents and low *p*H to elute proteins. In an organic solvent, the surfaces of both proteins and RPC sorbents are solvated. In the displacement model, when a protein adsorbs to a hydrophobic sorbent, the organic solvent is displaced from the contact surface areas of both the protein and sorbent. Although the same equation applies to both IEC and RPC, the displacement process in RPC is slightly different than IEC. In RPC, Z is the sum of solvent molecules that are displaced from both the protein and the sorbent. Although Z has a slightly different meaning in RPC, Z values of proteins are generally five to ten times higher in RPC than IEC. The practical implications of this will be discussed below.

There is little relation between the concentration of hydrophobic amino acids in a polypeptide and either its Z value or chromatographic retention in RPC (*5–7*). In addition, efforts to predict the retention of peptides in RPC on the basis of group-specific contributions fail when the peptide is larger than 20 residues (*8*). Thus, three-dimensional structure in polypeptides sterically limits the contact of amino acids with the sorbent surface in RPC.

Displacement phenomena also occur in hydrophobic interaction chromatography (HIC). HIC is carried out on weakly hydrophobic columns in high concentrations of salt such as ammonium or sodium sulfate. Under these conditions there is substantial hydration of both the weak hydrophobic coating on the column and the surface of the protein (*9*). The very high surface tension of these solutions provides sufficient driving force so that hydrophobic groups on the external surface of a protein interact with the weakly hydrophobic surface of the column (*10*). When this occurs, both water and salt are displaced from the protein-sorbent interface (*11*). This displacement process has been coupled to variations in the three-dimensional structures of lysozyme, β-lactoglobulin A, and cytochrome c.

These observations in IEC, RPC, and HIC systems lead to several conclusions. The first is that macromolecular species may interact with chromatographic sorbents through multiple residues. This means that before elution, such molecules must be desorbed from surfaces at many sites. The second conclusion is that Z has a strong influence on the conditions required for desorption. Because Z is exponentially related to k', very small changes in $[D_o]$ will have a large impact on k' when Z is large. The gradient elution model of Snyder (*12*) indicates that larger Z values make it possible to resolve proteins on columns of 5 to 10 cm in length. This effect is most prominent in RPC where Z values range from 20 to more than 200. The third major conclusion that may be drawn from these observations is that, in molecules with any degree of three-dimensional structure, only a portion of the functional groups are acting simultaneously to control chromatographic behavior.

Residues Controlling Retention

The fact that only those amino acid residues at or near the external surface of a protein may interact with another protein or surface has been extensively documented. Conversely, those amino acids buried in the interior of the three-dimensional matrix of the protein will play no direct role in surface interactions. Variant proteins that result from mutations, expression errors, or some type of post-translational modification can only be differentiated from native proteins by surface-mediated separation techniques if the structural alteration changes the three-dimensional structure of the protein or appears on the external surface. This is important in the separation of genetically engineered proteins because such variants occur at a higher rate than in natural systems (*13*).

The Chromatographic Contact Region

It has been shown in the protein-ligand interactions on which bioaffinity chromatography is based that specific areas or regions on the surface of proteins such as enzymes (*14*), hormones (*15*), antibodies (*16*), and antigens (*17*) determine their chromatographic behavior. Generally, the protein surface area involved in the adsorption process is no more than a few hundred square angstroms. In an enzyme, for example, chromatographic behavior is determined by amino acids in the active site (*18*). The alteration of a single amino acid or slight changes in the spatial orientation of amino acids within this region can have a major impact on adsorption. Antibodies can be even more specific. Because of biological recognition within the very small region of the epitope, immunosorbents can effect enormous purification of proteins (*19*). Other forms of chromatography are less specific. The degree to which they are less specific can be evaluated through the use of variant proteins.

The concept of using variant proteins to study the relation between protein structure and chromatographic behavior is simple. An amino acid substitution in a protein should influence chromatographic behavior only when it is in an area of the solute that makes direct contact with the chromatographic sorbent or when it alters the structure of the protein sufficiently to change the chromatographic contact region. Almost identical techniques have been used in epitope mapping (*17*). With the availability of numerous natural variants and the ability to produce an unlimited number of synthetic variants through recombinant DNA technology, it is possible, by a mapping process, to locate regions on the exterior of a protein that determine chromatographic behavior.

There are seven variants of avian lysozyme, all of which have nearly identical structure by x-ray crystallography (*20*). When these isoenzymes are examined by HIC, one particular region of the molecular surface opposite the catalytic cleft dominates chromatographic retention (*21*). This area extends from residue 41 to 102 and from residue 75 to the α-helical region that starts with residue 89. Amino acid substitutions within this region alter chromatographic behavior, whereas a substitution in other external faces of the protein has no influence on chromatographic retention. However,

the chromatographic contact region is sufficiently broad in HIC that six of the seven variant lysozymes may be resolved. Retention in other chromatographic modes may also be dominated by a specific portion of the protein surface. For example, chromatography of hen egg white lysozyme on immobilized metal affinity columns is influenced by histidine, and there is only one histidine at residue 15. In contrast, cation-exchange behavior of the lysozyme mutants is determined by a substantial portion of the surface (*22*). Histidine substitutions at residues 15, 41, 77, 103, and 114 show that all of these positions are involved in IEC retention.

IEC retention studies point to the reason for these variations in surface involvement in the different modes of chromatography. The amphoteric nature of proteins makes their retention in ion-exchange systems quite different from that in other chromatographic systems. It seems that proteins should not adsorb to ion-exchange columns at their isoelectric points, where the net charge is zero. However, this is often not the case. For example, β-lactoglobulin can be adsorbed to an anion-exchange column through three to four negative charges when the molecule has a net positive charge of 3 or more (*23*). The same phenomenon is seen with cytochrome c (*22*). In fact, there is little relation between the *Z* number of a protein on an ion-exchange column and its net charge. This implies that there may be regions of charge localization on the protein surface that are quite different from the net charge of the molecule. The structures of both β-lactoglobulin and cytochrome c indicate that this is true.

These observations lead to the conclusion that when there is a heterogeneous distribution of functional groups on the protein surface, those regions with the highest concentration of functional groups involved in the chromatographic process can dominate retention. In contrast, when there is little localization of functional groups on the surface of a protein, such as in lysozyme and subtilisin, it is to be expected that a relatively large portion of the surface will determine chromatographic behavior.

Amphipathic helices provide another case of asymmetric amino acid distribution determining a specific chromatographic contact region in a polypeptide (*24*). Because there is an accumulation of hydrophobic amino acids on one side of an amphipathic helix, that region of the molecule will dominate retention in the hydrophobic interaction modes of chromatography.

A study (*25*) with lactate dehydrogenase isoenzymes shows that in addition to the two extremes noted above, that is, a single dominant chromatographic contact region or no dominant contact region, multiple subunits or domains in a protein may play a role in its chromatographic behavior. At *p*H 7.2, the lactate dehydrogenase isoenzymes elute from an anion-exchange column in the order M_4, M_3H, M_2H_2, MH_3, and H_4, with *Z* numbers of approximately 0, 3, 6, 9, and 9, respectively. Under these conditions only the H subunit has an affinity for the anion-exchange matrix.

Because there is a tetrahedral arrangement of subunits in these isoenzymes and only three of the subunits can simultaneously contact the surface of an anion-exchange sorbent, these results indicate that each H subunit interacts with the sorbent through an average of three charges. Because only three subunits can contact the surface simultaneously in both MH_3 and H_4, steric limitations dictate that they will both have *Z* numbers of 9. Separation of these two isoenzymes is apparently effected by the existence of multiple orientations of H_4 that are energetically equivalent. These multiple possibilities for adsorption would cause more H_4 to be adsorbed than MH_3 at any time and would increase its chromatographic retention. From these results on lactate dehydrogenase it may be concluded that (i) regions on the surface of a protein that interact with a column may be contributed by multiple subunits, (ii) these regions need not be contiguous, (iii) functional groups involved in the adsorption process may be spread across large areas of the protein surface, and (iv) there may be multiple equivalent contact sites on the surface of a protein.

Mode-Specific Contact Regions

I suggested above that the distribution of amino acids on the surface of a protein is important in determining the chromatographic contact region. A highly asymmetric distribution would produce a specific contact region as opposed to a uniform distribution, which would cause all areas of the surface of the molecule to be involved equally in the adsorption process. The asymmetric distribution of amino acids even applies to classes of amino acids. For example, hydrophilic and hydrophobic amino acids are segregated in amphipathic proteins. Extending this logic to a comparison of the various chromatographic modes, one would expect the chromatographic contact regions of these proteins to be localized on different portions of the protein surface. It was noted above that for lysozyme the chromatographic contact region for HIC is on the side of the molecule opposite the catalytic cleft. Thus, bioaffinity and HIC columns interact with different portions of the lysozyme surface. In addition, portions of the surface outside of both the HIC contact region and the active site have been implicated in electrostatic adsorption. The histidine at residue 41 is in such a region. An immunosorbent column prepared with an antibody targeted for the Arg^{125}, Arg^{5}, Glu^{7}, Arg^{14}, and Lys^{13} epitope (*26*) would probe still another portion of the molecule. Although the results above dealt with a specific case, lysozyme, the findings are probably general. Therefore, the portion of the protein surface probed in a given chromatographic mode will be protein specific.

Conformational Changes and Chromatographic Behavior

Under physiological conditions proteins generally have a preferred conformational state. Unfortunately, nonphysiological conditions must often be used during protein isolation. Thus, the protein of interest may be denatured and require refolding to regain the native structure. Although refolding is easily achieved with small polypeptides, it can be a formidable task with large proteins and polypeptides that have undergone post-translational modification. Obviously it is best to prevent denaturation during chromatographic separations if possible. If not, it is necessary to understand how denaturation occurred and to evaluate its impact on chromatographic behavior.

Both hydrophobic and coulombic forces are important in the maintenance of protein structure. The same forces are used to effect chromatographic separations. In essence, the column is competing in the contact region with the rest of the protein structure for functional groups at the interface. When competing hydrophobic and coulombic forces on the column are greater than those maintaining protein structure, denaturation can occur. Mobile phases can also trigger denaturation. Strongly retained solutes often require mobile phase additives that disrupt intramolecular hydrophobic and coulombic forces beyond the chromatographic contact region. Denaturation may occur on all types of liquid chromatography columns.

The problem of denaturation is greatest in RPC where both organic solvents and very acidic mobile phases must be used to elute proteins. Because hydrophobic forces play a major role in organizing and maintaining the three-dimensional structure of a protein, the presence of alkyl silane residues of the column, organic solvents in the mobile phase, and very acidic conditions can denature the

protein. Multimeric proteins with dissimilar subunits present a second problem. Denaturation of such a protein on the column could cause dissociation and separation of the subunits. This would make it difficult to locate the subunits and reassemble the native protein. The problem of disrupting quaternary structure even extends into the analytical mode. Protein denaturation can increase sample complexity to the point that it is impossible to differentiate between sample components and artifacts.

The external hydrophobicity of a protein in the native state will be quite different from that in the unfolded state because hydrophobic side chains are exposed during denaturation (*27–29*). Direct spectral measurements indicate that when lysozyme contacts an RPC sorbent, there is a conformational change in the chromatographic contact area of the protein (*30*). As soon as the protein is adsorbed onto the surface, some tryptophans of the lysozyme appear to become rapidly exposed to solvent. Rate constants for this transition determined by spectral and chromatographic measurements are in good agreement. It is even possible in denaturing mobile phases that a protein can be folding and unfolding during the chromatographic process (*31*). The rate of this process determines whether one will obtain predominantly native protein, denatured protein, or a mixture of conformers. Interconversion of conformers in the chromatographic system can also influence peak shape. In both HIC (*11*) and RPC (*31*), the tendency is to go from a sharp peak in the native state, through a broad peak in the equilibrium system, to a sharp peak in the denatured state. By increasing column temperature, it has been shown with ribonuclease A that the size of the sharp peak of denatured protein increases at the expense of the broad peak (*32*). In general, chromatographic retention increases with denaturation in RPC and HIC. It has been shown by diode-array spectroscopy that in broad peaks there may be a variation in conformer composition across the peak (*11*). It is desirable to choose chromatographic conditions that select a single conformational state for separation.

Even though protein structure is altered in RPC, substantial secondary and tertiary structure can be retained. Evidence for this conclusion is based on observations that (i) more than one peak can be observed for a single protein, meaning that there are multiple conformational states and differing degrees of unfolding (*29, 33*), (ii) retention cannot be predicted by a simple summation of the group increments of individual amino acids in the polypeptide (*33*), (iii) the incremental contribution to retention of a functional group depends on its position in the polypeptide (*7*), (iv) there is little relation between Z values and molecular weight in nondenaturing systems (*6, 7*), (v) Z number increases with more severely denaturing conditions (*4*), and (vi) spectral techniques show the retention of protein helical content on the sorbent surface (*34*).

With both proteins and peptides, the addition of organic solvents during RPC under acidic conditions produces a much greater disruption of tertiary and quaternary structure than of secondary structure (*34, 35*). This is because hydrophobic forces are major contributors to tertiary and quaternary structure and hydrogen bonds are more stable in organic solvents than in water. Maintenance of tertiary structure during RPC is generally associated with disulfide-bonded polypeptides.

The contribution of disulfide bonding to chromatographic behavior in RPC has recently been studied with interleukin-2 (IL-2) variants that were substituted at positions 1, 58, 104, 105, and 125 (*7*). Natural IL-2 has a single disulfide bridge between the cysteines at positions 58 and 105, which forms spontaneously as the protein folds. The molecule can also be forced to form disulfide bonds at positions 58 and 125 or 105 and 125. Although all the IL-2 species examined were of approximately the same molecular weight (15,000) and relative hydrophobicity, their Z numbers varied by a factor of 2.5. This is apparently due to large conformational differences in these molecules and in their chromatographic contact regions. The IL-2 species with unnatural disulfide bridges had a much larger Z than the native species. These results were interpreted to mean that the external hydrophobicity of the unnatural species was substantially greater than that of the natural species.

The influence of three-dimensional structure on chromatographic retention can also be seen in the oxidation of methionine in IL-2 (*7*). Although there are four methionines in the molecule, only the oxidation of Met^{104} to methionine sulfoxide can be observed by RPC. When any of the IL-2 species is converted to a molecule with an unnatural disulfide bridge, it is impossible to distinguish even this methionine sulfoxide. This suggests that the conformational state in the unnaturally disulfide-bridged species does not allow Met^{104} to come in contact with the sorbent surface.

The temperature at which polypeptides undergo denaturation on HIC and RPC columns is almost always lower than in solution (*28, 33, 35*). This behavior has been attributed to the nature of the hydrophobic stationary phases themselves. Since tertiary and quaternary structure are stabilized by hydrophobic interactions, the possibility of denaturation must always be taken into consideration when a hydrophobic column is used. In fact, the alkyl silane stationary phase of an RPC column is a more powerful denaturant than the organic solvents used to elute the protein (*35*).

The effects of both urea and thermal denaturation on the cation-exchange chromatographic behavior of α-chymotrypsinogen A and lysozyme have been found to produce a sharp decrease in chromatographic retention over a narrow temperature range (*36, 37*). Although the Z number of α-chymotrypsinogen A increased upon denaturation, the net affinity of the protein for the sorbent decreased. Under certain conditions, native and denatured forms were found to be in equilibrium on the column.

Implications

Large-scale production of genetically engineered proteins has fostered the need for both high-resolution analytical techniques and large-scale purification systems. Because protein synthesis in genetically engineered organisms is often accompanied by both expression errors and incomplete post-translational processing, the heterogeneity introduced during synthesis may make these proteins more difficult to purify than the natural species. The task of proving that genetically engineered proteins are either identical or structurally equivalent to wild-type proteins, of equal or higher biological activity, and free of impurities will be a major activity of both the biotechnology industry and regulatory agencies. Chromatography will play an essential role in the future of biotechnology. As a consequence, a better understanding of chromatographic behavior and the limits of chromatographic resolution is needed.

Although our knowledge of the relation between chromatographic behavior and protein structure is primitive, it is already possible to begin to understand the limits of chromatographic systems. On the basis of the discussions above, several broad conclusions may be drawn concerning the ability of liquid chromatographic systems to discriminate among closely related polypeptides. The first conclusion is that chromatographic behavior is dominated by those amino acids and oligosaccharides that contact the sorbent surface. This is particularly significant when comparing bioaffinity and other forms of chromatography. A small area on the surface of a biological macromolecule generally determines chromatographic behavior in bioaffinity chromatography. In contrast, much larger regions of the surface determine chromatographic behavior in most of the other modes of chromatography.

The second conclusion is derived from the first. Any change in

molecular structure outside the chromatographic contact region, that is, either the exterior or interior, will go undetected unless this change alters the structure of the chromatographic contact region. Although liquid chromatographic systems are capable of remarkable discrimination, their greatest weakness is the prerequisite that differences must lie within the chromatographic contact region to be detected.

These conclusions have important implications in both preparative and analytical systems. For example, it would be possible to purify most of the lysozyme variants with a single monoclonal antibody column because the chromatographic contact region of the immunosorbent is narrowly targeted and amino acid variations are broadly distributed. HIC and IEC columns, in contrast, are less effective in the initial purification of lysozymes but can easily differentiate between most of the lysozyme variants.

The case of lysozyme variants illustrates that purification of a genetically engineered protein based only on immunosorbents is probably not wise in systems where expression errors, faulty post-translational processing, and chemical alterations during purification can be encountered. In particular, monoclonal antibodies will be ineffective in discriminating among these small, random variations within a structure. Analytical systems based on immunological assays will be plagued with the same problem. Although chromatographic modes such as IEC, HIC, and RPC are much less dramatic than immunosorbents, they are less expensive and can play an important role in molecular discrimination.

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High-Speed Chromosome Sorting

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Dual-beam high-speed sorting has been developed to facilitate purification of chromosomes based on DNA staining with the fluorescent dyes Hoechst 33258 and chromomycin A3. Approximately 200 chromosomes per second of two types can be sorted from a suspension of chromosomes isolated from human lymphoblasts while fluorescent objects (chromosomes, debris fragments, chromosome clumps, and nuclei) are processed at the rate of about 20,000 per second. This sorting rate is approximately ten times that possible with conventional sorters. Chromosomes of a single type can be sorted with a purity of about 90 percent. DNA from the sorted chromosomes is suitable for construction of recombinant DNA libraries and for gene mapping.

CHROMOSOMES HAVE BEEN RECOGNIZED FOR ALMOST A century as the fundamental organizational units of DNA and as the determinants of gene segregation during cell division. Much effort has been devoted to determination of the chromosomal locations of specific genes and to identification of disease-linked chromosome aberrations in humans. Over 1600 DNA sequences have now been mapped, at least provisionally, to chromosomes. In addition, the chromosomal locations of more than 50 disease-linked DNA sequences are known (*1, 2*). Efforts to understand the DNA sequence organization of chromosomes are now accelerating with the initiation of efforts to determine the complete genetic structure of the human genome (*2*). Much of this work can be facilitated by the ready availability of DNA from one or

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