ACCESSIBLE HYDROPHOBIC GROUPS OF NATIVE PROTEINS

B.H.J. Hofstee
With the technical assistance of N. Frank Otillio
Biochemistry Division, Palo Alto Medical Research Foundation
Palo Alto, California 94301

Received February 7,1975

SUMMARY. Evidence is presented that at least eleven out of thirteen arbitrarily chosen proteins are endowed with accessible hydrophobic groups capable of hydrophobic "bond" formation with n-hexylamine-substituted agarose.

It may be assumed that the hydrophobic amino acid side chains generally are found in the interior of a protein molecule whereas the hydrophilic side chains, in particular the charged ones, are located on the outside. In fact, the formation of the hydrophobic core is considered an important factor in determining the native structure of a protein (1). Nevertheless, an examination by Klotz (2) of several proteins of which the three dimensional structure is known, showed that hydrophobic groups occur much more frequently on the surface of these proteins than had been assumed. This not only pertains to small hydrophobic groups such as the methyl group of alanine, but often includes the most strongly hydrophobic side chains such as those of phenylalanine and tryptophan as well.

Although through an entirely different approach, not requiring detailed knowledge of the three dimensional protein structure, the present results provide evidence for the occurrence of hydrophobic groups on the molecular surface of many proteins in addition to those investigated by Klotz. For this purpose the extent of binding of the proteins on columns of agaroses substituted with hydrophobic ligands was determined in the presence of 3.3 M NaCl. The salt served to enhance hydrophobic "bond" formation and at the same time to quench electrostatic effects (3-6).

EXPERIMENTAL

The adsorbents were prepared by substitution of CNBr-activated agarose (Sepharose 4B) with n-hexylamine or with phenylalanine (7,8),

followed by exhaustive washing of the final product with a mixture of 50 per cent ethylene glycol and 1 M NaCl in 0.01 M Tris-HCl buffer, pH 8. As determined by Ponceau binding or by titration (4), the initial ligand concentrations on the two adsorbents were at least 3 μMol/ml gel. The proteins were highly purified commercial preparations purchased from CalBiochem., Mann Research, Nutritional Biochem. Corp. or Worthington.

Binding studies were performed with a few mg of a protein dissolved in 5 ml of the buffer-salt solution and applied to 25 ml of an adsorbent contained in a 50 ml column filled with supporting siliconized glass beads of 6 mm diameter in order to counteract excessive packing (9). The jacketed columns were equilibrated at ≈ 5° with 3.3 M NaCl in 0.01 M Tris-HCl buffer, pH 8. After application of the protein, 2-3 bed volumes of the salt-buffer solution were pumped through and the appearance of protein, if any, in the filtrate was monitored by UV absorbance or by fluorescence. Subsequently a solution of 50 per cent ethylene glycol and 1 M NaCl in buffer was applied to the column and the protein content of the filtrate was monitored again.

RESULTS

It was noted that most often all or part of a protein either appeared in the filtrate with little or no retardation (Fraction I) or remained adsorbed after at least 2-3 bed volumes of the salt solution had passed through the column. In the latter case immediate release of the protein (Fraction II) was achieved with buffer containing 50 per cent ethylene glycol and 1 M NaCl, confirming that such a fraction was hydrophobically bound (10). The absence of a protein in Fraction I and its occurrence in Fraction II only, is indicated by a + sign in Table I. Conversely, a - sign indicates that a protein appears only in Fraction I and none in Fraction II. The occurrence of part of the same protein preparation in both fractions is indicated either by $\pm$ or $\mp$, depending on which fraction was largest. A dashed sign refers to a minor component of a few per cent or less. It would seem unlikely that the apparent gross chromatographic inhomogeneity displayed by several of these highly purified protein preparations is due to contamination with other proteins. As suggested previously, apparent inhomogeneity with respect to binding by this type of adsorbent could be the result of inhomogeneity of the adsorbent binding sites in conjunction with "irreversible" multiple point binding of the protein (4,10).

The agarose-bound amino groups of the adsorbents carry a positive

PROTEIN	AGAROSE SUBSTITUENT	
	n-HEXYLAMINE	L-PHENYLALANINE
SERUM ALBUMIN	+	∓
OVALBUMIN	∓	∓
α-LACTALBUMIN	∓	∓
β-LACTOGLOBULIN	+	∓
7 S γ-GLOBULIN	+	+
CHYMOTRYPSINOGEN	+	−
α-CHYMOTRYPSIN	±	∓
TRYPSINOGEN	±	∓
THROMBIN	+	+
DN-ase	±	∓
RN-ase	±	±
CYTOCHROME c	∓	∓
MYOGLOBIN	∓	∓

TABLE I

Protein Binding by Substituted Agaroses
in the Presence of 3.3 M NaCl at pH 8

charge (11). For A-Phe* the charge is neutralized by the ionized carboxyl group of the amino acid. Furthermore, the hydrophobicity of a phenyl group corresponds to that of only 3-4 straight chain hydrocarbons (see ref. 12). Thus the total hydrophobicity of the $-CH_2$-phenyl side chain of Phe is equivalent to 4-5 methylene groups, i.e., less than that of the C_6-ligand.

The data of Table I show that in the presence of 3.3 M NaCl, binding by the positively charged C_6-adsorbent occurs with negatively charged proteins such as BSA, β-LG and DN-ase as well as with positively charged

*The abbreviations used are:

Phe, phenylalanine; C_6, n-hexylamine; A-X, agarose substituted with X; BSA, bovine serum albumin; β-LG, beta-lactoglobulin; DN-ase, deoxyribonuclease I; RN-ase, ribonuclease.

ones such as chymotrypsinogen and RN-ase. The same holds true for the (neutral) A-Phe adsorbent. Also, the relatively low affinities of A-C_6 for ovalbumin and α-lactalbumin as compared to serum albumin, correspond to the behavior of these proteins on a similar but uncharged hydrophobic adsorbent (13). These observations indicate that charge effects are quenched at the high salt concentration that was employed. Furthermore, although several of the proteins bound by A-C_6 are not bound or are less strongly bound by the less hydrophobic Phe-ligand, all of the proteins bound by A-Phe also are bound, and most often more strongly, by the C_6-adsorbent. Thus the evidence indicates that the difference in binding of different proteins primarily is a matter of hydrophobicity without electrostatic effects playing a major role. The data also suggest that little or no "specificity" is involved. If specificity did play a role, at least some of the proteins might show more extensive binding by A-Phe than by A-C_6, which does not appear to be the case. The fact that increasing the hydrophobicity of the adsorbent, i.e., from A-Phe to A-C_6, does not have the same effect on all of the proteins of Table I, merely may be the result of the difference in their hydrophobicities. Under the experimental conditions the A-Phe adsorbent may be sufficiently hydrophobic for the binding of certain proteins (e.g., 7S γ-globulin or thrombin) whereas for others (e.g., BSA or chymotrypsinogen) a more hydrophobic adsorbent such as A-C_6 is required.

The finding that some proteins, for instance, ovalbumin and myoglobin show little affinity, even for A-C_6, not necessarily indicates that they are devoid of hydrophobicity. It is possible that binding of these proteins requires ligands that are more strongly hydrophobic and/or that the binding should be carried out in the presence of salts with stronger "structure forming" properties in order further to enhance hydrophobic binding (14,15). In general, experimentation with extremely hydrophobic ligands is avoided because of possible "detergent" action and the

exposure of internal hydrophobic protein groups. On the other hand, in the case of n-alkyl sulfates, even the decyl derivative seems to bind to serum albumin without significant conformational change of the latter (16,17), despite evidence that the internal binding in serum albumin is unusually weak (18). Furthermore, the presence of "structure forming" salt which enhances intermolecular hydrophobic bonding between ligand and protein, also would tend to stabilize the hydrophobic interior of the protein. Therefore, it may be assumed that with the relatively small hydrophobic ligands employed and particularly at the relatively low temperature, the proteins remained in the "native" state. Thus, the data of Table I present evidence that with the possible exception of ovalbumin and myoglobin, the native form of all of the proteins involved are endowed with accessible hydrophobic groups, which under the proper conditions are capable of hydrophobic bond formation with immobilized hydrophobic ligands.

DISCUSSION

Earlier suggestions as to the separation of proteins by "hydrophobic affinity chromatography" (10) were largely based on the belief that the occurrence of external hydrophobic groups is relatively rare and on the tacit assumption that such accessible hydrophobic groups usually are connected with a specific biological function. Subsequent evidence indicated, however, that a number of arbitrarily chosen enzymes are bound by the present type of adsorbent through sites other than the enzyme active center (3). The present results further confirm the general occurrence and "non-functional" nature of accessible hydrophobic sites on proteins. Such sites, nonetheless, could play a role in the binding of the proteins by intracellular matrices. Although for most proteins hydrophobic binding by a ligand as small as n-hexyl probably would not occur at physiological salt concentrations, the requirement for extremely high salt concentrations rapidly diminishes with increas-

ing size of the hydrophobic ligand (4,19).

For the chromatographic separation of proteins, the hydrophobic factor might present a parameter of equal importance and range of applicability as electric charge and molecular size, employed respectively in ion-exchange and exclusion chromatography. Separation on the basis of differential adsorption on a series of columns of varying hydrophobicities generally is preferred over differential elution from a particular adsorbent, such as elution by a gradient of increasing concentrations of a chaotropic agent. A procedure using a hydrophobicity gradient of interconnected columns of adsorbents of increasing hydrophobicity has recently been proposed (20). The protein mixture is applied to the first, least hydrophobic, adsorbent and is washed into the series by means of the ambient medium. In this manner each protein tends to be bound by the adsorbent that provides the minimum degree of hydrophobicity required and elution of the subsequently disconnected columns is more likely to be achieved with a relatively mild eluant. By contrast, the use of a single arbitrarily chosen adsorbent has the disadvantage that the hydrophobicity may be too low for a certain protein and too high for another, i.e., the former may not be bound at all, whereas the latter possibly is bound too strongly to allow elution without denaturation (for details and preliminary results on the separation of blood proteins, see ref. 20).

REFERENCES

1. Kauzman, W. (1959) Adv. Prot. Chem., 14, 37-47.
2. Klotz, I. M. (1970) Arch. Bioch. Biophys., 138, 704-706.
3. Hofstee, B.H.J. (1973) Bioch. Biophys. Res. Comm., 53, 1137-1144.
4. Hofstee, B.H.J. (1974) Immobilized Biochemicals and Affinity Chromatography (R. B. Dunlap, ed.), pp. 43-59, Plenum Publ. Corp., New York.
5. Porath, J., Sundberg, L., Fornstedt, N., and Olsson (1973) Nature, 245, 465-466.
6. Hjertén, S. (1973) J. Chromatogr. 87, 325-331.
7. Axén, R., Porath, J. and Ernbäck, S. (1967) Nature 214, 1302-1304.
8. Cuatrecasas, P. (1970) J. Biol. Chem., 245, 3059-3065.
9. Sachs, D. H. and Painter, E. (1972) Science 175, 781-782.

10. Hofstee, B.H.J. (1973) Anal. Bioch. 52, 430-448.
11. Porath, J. (1968) Nature, 218, 834-838.
12. Hofstee, B.H.J. (1958) Arch. Biochem. Biophys., 78, 188-196.
13. Jost, R., Miron, T. and Wilcheck, M. (1974) Bioch. Biophys. Acta, 362, 75-82.
14. von Hippel, P. H. and Schleich, T. (1969) Structure and Stability of Macromolecules (S. N. Timasheff and G. D. Fasman, eds.), pp. 417-574, Marcel Dekker, New York.
15. Dandliker, W. B. and de Saussure, V. A. (1971) The Chemistry of Biosurfaces (M. L. Hair, ed.), pp. 1-43, Marcel Dekker, New York.
16. Reynolds, J. A., Herbert, S., Polet, H. and Steinhardt, J. (1967) Biochemistry 6, 937-947.
17. Tanford, C. (1973) The Hydrophobic Effect: Formation of Micelles and Biological Membranes, pp. 137-138, Wiley-Interscience, New York.
18. Tanford, C. (1961) Physical Chemistry of Macromolecules, p. 518, John Wiley and Sons, New York.
19. Hofstee, B.H.J., Polymer Grafts in Biochemistry (H. F. Hixson, ed.), Marcel Dekker, in preparation.
20. Hofstee, B.H.J., Preparative Biochem., in press.