On the Prevalence of "Nonspecific" Binding at the Specific Binding Sites of Globular Proteins*

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Abstract. Strong binding of dyes to simple globular proteins takes place predominantly in areas overlapping the binding sites for substrates, coenzymes and prosthetic groups, in preference to other regions of the protein surface. The structure of the dyes bears no obvious relationship to that of the normal ligands. It is proposed that this phenomenon is a reflection of the special stereochemical features of such sites, their hydrophobicity relative to other portions of the protein surface, and, possibly, greater flexibility in these regions of the protein molecule. The binding properties of antibodies and bovine serum albumin are discussed in relation to this apparent versatility of protein binding sites towards structurally unrelated organic ligands.

A number of years ago, a systematic examination of enzyme-dye interactions was undertaken in this laboratory in the hope that highly specific protein-dye complexes could be detected. This approach was based on the fact that the surfaces of globular protein molecules display complex asymmetric arrangements of side-chains, and that such surfaces would present only a very limited number of sites at which suitably chosen rigid, nearly planar, dye molecules could achieve sufficient contacts for tight binding. The choice of proteins for this study was largely one of convenience based on their ready availability in large quantities. The proteins chosen were trypsin, chymotrypsin and their zymogens, Novo and Carlsberg subtilisins, papain, lysozyme, ribonuclease A, and cytochrome c. The interaction of these proteins with fifty dyes, belonging to the azo, acridine, phenothiazine, cyanine, quinone-imine, and anthraquinone classes, was examined by spectrophotometry and equilibrium dialysis. Dyes possessing a net charge exceeding two at neutral pH were avoided, and pH and buffer composition were chosen to minimize interactions due largely to electrostatic attraction. The studies were performed under conditions expected to select for strong interactions. Dye concentrations were below 5×10^{-4} M and a molar excess of protein was used. The above screening procedure led to the demonstration of 1:1 interactions of thionine, Biebrich Scarlet, and 4-(4'-aminophenylazo)phenyl-arsonic acid with trypsin, chymotrypsin, and the subtilisins, respectively.[1, 2] These protein-dye interactions were highly specific and dyes of closely related structure were either not bound or bound very weakly. Since the dyes bound to the proteolytic enzymes were not bound by the zymogens,[1] and were dis-
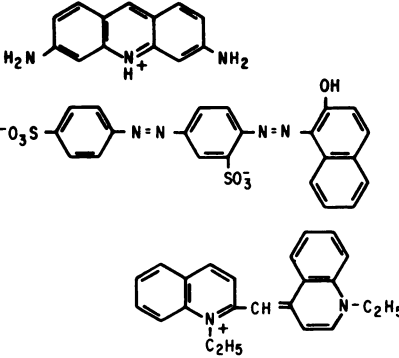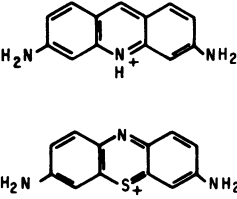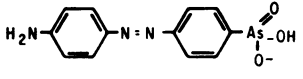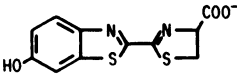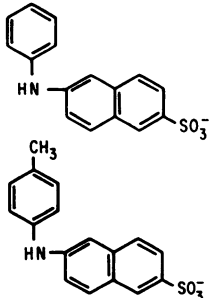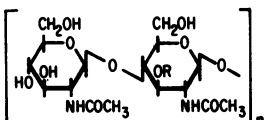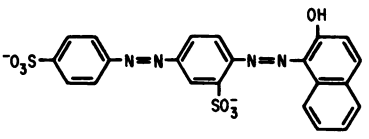
ENZYME	SUBSTRATE	DYE

FIG. 1.—One-to-one enzyme-dye complexes.   See text for references.

placed by competitive inhibitors, and substrates, as well as specific reagents which modified either the serine or histidine residues at the active sites of these enzymes,[1, 2] it was concluded that the strong dye-binding sites all involve, at least in part, the area of the protein molecule concerned with substrate-binding and catalysis. A remarkable feature of the investigation, however, was a failure to find other strong interactions, either at multiple or single sites with the various native proteins and dyes studied.[3] This suggested that strong binding sites on proteins (characterized by a K_{diss} of 10^{-5} M or lower) for organic ligands of the type studied are rare. A striking aspect of the strong protein-dye interactions

FIG. 2.—One-to-one protein-dye complexes. See text for references.

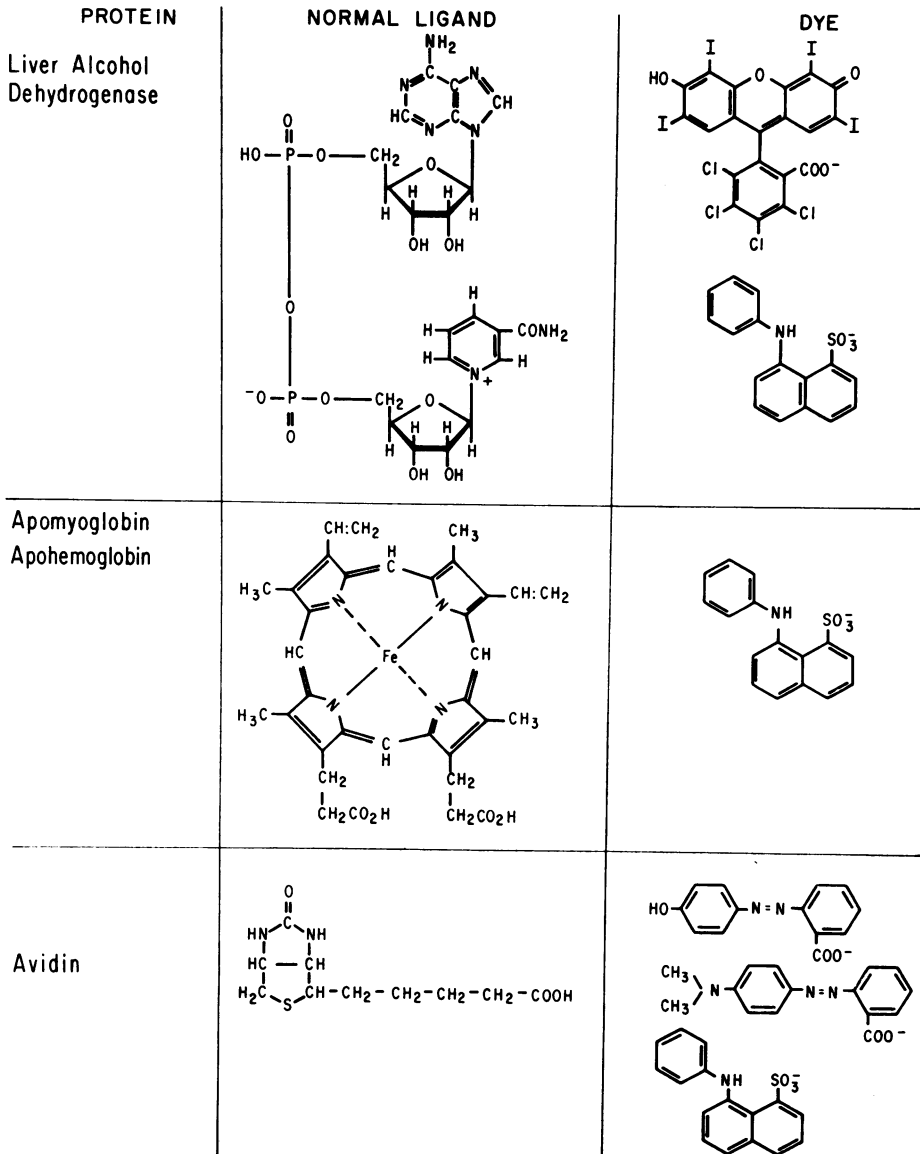observed[1-3] was that in every case the structure of the dye bore no obvious relationship to those of the substrates of the enzymes studied (see Fig. 1). Since our study was limited to a relatively small number of proteins, a review of the literature was undertaken to examine the generality of the above observations. This review showed that relatively few studies of enzyme-organic ligand interactions had been performed with ligands which were not substrate (or cofactor) analogs. Further, in a large number of studies only spectrophotometric criteria of binding were employed—so that the presence of sites at which binding may have occurred, with little or no perturbation of the spectrum of the ligand, may well have been overlooked. However, some additional interesting studies could be added to extend our observations. As shown in Figures 1 and 2, 1:1 protein-dye complexes have been obtained also at the active sites of luciferase[4] and lysozyme,[5] at the prosthetic group binding sites of apomyoglobin and apohemoglobin,[6] the coenzyme binding site of liver alcohol dehydrogenase,[7] and the biotin-binding site of avidin.[8] In all cases, strong interaction took place uniquely at the binding sites. No secondary sites were detected. Since in every case the dyes bound at these sites (Figs. 1 and 2) bore little structural resemblance to each other or to the normal ligands, these studies support the conclusion reached on the basis of our limited investigations, that substrate, prosthetic group, or coenzyme binding sites on globular proteins provide a uniquely favorable environment for interaction with a variety of organic molecules.[3]

Three exceptions to the above observations have been reported. One-to-one complexes, slightly weaker than those discussed above, have been observed for chymotrypsin with thionine,[1] and 2-P-toluidinylnaphthalene-6-sulfonate,[9] and for ficin with proflavine.[10] Ternary complexes of protein, dye and small substrates (or inhibitors) are observed in these cases. With the last two of these, the enzyme-dye complexes show altered kinetic behavior, and thus it is possible that the dyes are indeed bound in areas of the polypeptide binding sites of these enzymes removed from the immediate vicinity of the catalytic site.

Several lines of evidence indicate that the active site region in a number of enzymes possesses unique features. X-ray diffraction studies on several enzymes have shown that the active site regions appear to take the form of a cleft or a depression (see ref. 11 for a review). Clearly such regions would afford a greater number of possible modes of binding to a rigid organic molecule than would other areas of the protein surface. Indeed, different modes of binding of substrates have even been demonstrated, leading to productive and nonproductive complexes, as seen in the case of lysozyme[12] and carboxypeptidase *A*.[13] X-ray crystallographic studies on a variety of protein-ligand complexes,[11] have indicated that hydrophobic bonding, hydrogen bonding, and electrostatic interactions, all play an important role in the formation of such complexes. It might be expected, therefore, that molecules capable of participating in all of these types of interaction would represent the best potential ligands—and many dyes meet such criteria admirably.

It is generally recognized that organic molecules tend to associate in aqueous solution. This tendency, generally termed "hydrophobic bonding" is a conse-

quence both of the properties of the solvent, water, and of London dispersion forces between the solute and solvent molecules. This topic has been the subject of many recent discussions (see, refs. 14–17, for example). A number of general observations favor the active site of enzymes as a region of preferred organic molecule binding on the protein surface. Addition of small amounts of organic solvents, such as alcohols, acetonitrile, or dioxane, decreases the affinity of substrates for a number of enzymes.[18–24] The effect observed is in excess of that expected from the change in the bulk properties of the solvent, and, in the few cases which have been examined carefully, has been attributed in large measure to a competition by the organic solvent for the substrate binding site.[22, 23] Likewise, in studies of transesterification reactions with a number of different enzymes, invariably it has been observed that the order of effectiveness of normal alcohols in competing with water is amyl > butyl > propyl > ethyl > methyl.[25–29] These observations suggest that the concentration of the organic solvent is greater at the active site than in the bulk solvent, and that actual weak binding of the organic solvent is taking place in this region.[30] That this phenomenon is not a general property of the native protein surface is suggested by the work of Timasheff and Inouye,[31] who found no significant binding of ethylene glycol, or methoxyethanol, at low concentrations (<30%), to β-lactoglobulin. "Solvent perturbation" difference spectra of native proteins, above 270 mμ, produced by dioxane, or ethylene glycol, up to 20 per cent by volume, agree well with those obtained with the appropriate mixture of tyrosine and tryptophan derivatives.[32] This also suggests that the distribution of solvent components at most of the protein-solvent interface is grossly similar to that in the bulk solvent. These general considerations appear consistent with the view, proposed by various authors for a number of individual enzymes (see refs. 14, 23, 33, and 34, for example), that areas favoring hydrophobic interactions, are a common feature of the active site region of enzymes and, further, that this feature is not shared to a similar extent by other areas of the protein surface. This conclusion is satisfying from the standpoint of the considerations of thermodynamic stability of native enzymes in aqueous solution, and is compatible with the presently available relevant data on the three-dimensional structure of proteins.

The above discussion of the apparent versatility of the binding sites of proteins with respect to structurally unrelated ligands is relevant to several problems of current interest. For instance, a major question is that of the minimal number of different antibodies which have to be genetically specified to account for the observed, apparently inexhaustible capacity of the immune system to respond to challenge by an enormous range of antigens. Our studies with enzymes would predict that the binding sites of antibodies may show quite unsuspected "specificity" towards determinants totally unrelated to that in response to which they were formed. Fortunately, a relevant example has been reported. A carp anti-dinitrophenyl antibody was found to bind uracil "specifically" with reasonable affinity.[35] Thus it is likely that the actual number of different antibodies which an organism can synthesize may be significantly smaller than the number of antigens to which these antibodies can bind.

The unusual binding properties of serum albumin warrant special discussion.

More binding studies-involving serum albumin have been performed than the combined total of those with all other proteins.   An adequate review of the relevant literature is not attempted here.   However, certain pertinent observations should be indicated.   It is clear, from a host of studies, that serum albumin possesses only a limited number of strong binding sites for organic ligands, ranging from 1 to 5, depending on the ligand.   In analogy to the interactions of enzymes with alcohols, discussed above, the affinity of straight chain fatty acid anions for bovine serum albumin increases smoothly with increasing hydrocarbon chain length,[36] indicating the presence of significant areas available for nonpolar contacts at the binding sites.   The early studies of Klotz[37] already indicated that unrelated ligands compete for the strong binding sites.   A recent study of the binding of four structurally unrelated dyes to serum albumin showed clearly that these compete for the same binding sites.[38]   These studies suggest that the binding sites for organic ligands on the serum albumin molecule show to an extreme extent the binding versatility described above for the other types of binding sites, and that further competitive binding studies would be fruitful.

One aspect of protein-ligand interaction not examined above is that of the "conformational flexibility" of the protein molecule.   Changes in conformation, varying in extent, have been observed on binding of substrates and inhibitors to lysozyme,[12] ribonuclease,[11, 39] and carboxypeptidase A.[13]   The ease of deformation of the protein molecule at the active site region, relative to other areas of the protein surface, may contribute to the phenomena described above.

In conclusion, the above considerations do not imply that strong protein-dye (or other ligand) interactions do not occur in regions distant from binding sites, but that *interactions at binding sites occur with a far higher frequency*.   Likewise, the conclusions reached here are likely to be invalid for many weaker protein-ligand complexes characterized by K_{diss} values higher than $5 \times 10^{-4} M$.   The discussion also specifically excludes interactions of proteins with multiply charged ligands.   A major aspect of this contribution is to emphasize the versatility of the binding sites on proteins towards structurally unrelated ligands.

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