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Review

Supramolecularity creates nonstandard protein ligands[o]

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Congo red and a group of structurally related dyes long used to stain amyloid proteins are known to associate in water solutions. The self-association of some dyes belonging to this group appears particularly strong. In water solutions their molecules are arranged in ribbon-like micellar forms with liquid crystalline properties. These compounds have recently been found to form complexes with some native proteins in a non-standard way. Gaps formed by the local distribution of β-sheets in proteins probably represent the receptor sites for these dye ligands. They may result from higher structural instability in unfolding conditions, but also may appear as long range cooperative fluctuations generated by ligand binding. Immunoglobulins G were chosen as model binding proteins to check the mechanism of binding of these dyes. The sites of structural changes generated by antigen binding in antibodies, believed to act as a signal propagated to distant parts of the molecule, were assumed to be suitable sites for the complexation of liquid-crystalline dyes. This assumption was confirmed by proving that antibodies engaged in immune complexation really do bind these dyes; as expected, this binding affects their function by significantly enhancing antigen binding and simultaneously inhibiting C1q attachment. Binding of these supramolecular dyes by some other native proteins including serpins and their natural complexes was also shown. The strict dependence of the ligation properties on strong self-assembling and the particular arrangement of dye molecules indicate that supramolecularity is the feature that creates non-standard protein ligands, with potential uses in medicine and experimental science.

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Abbreviations: SRBC, sheep red blood cell; IgG, immunoglobulin G, TNP, trinitrophenyl.

The activity of proteins is generally correlated with binding of ligands. A special site, usually limited in size, is designed biologically for this purpose in native proteins. It may attach small monomeric molecules or suitable selected fragments of polymers. As a crevice available for permanent penetration in tightly packed protein molecules, the binding site is basically the only site which allows ligands to enter the low polarity portion of the protein. The usually tightly packed polar surface of proteins exposed to water is basically not capable of non-covalent binding of ligands because of the strong competition from water and ions. However, even occasional partial unfolding of the peptide chain makes the protein accessible for penetration and/or ligation by different molecules. Particularly the molten hydrophobic protein interior is known to bind many compounds of low polarity (Christensen & Pain, 1991; Creighton, 1992; Redfield *et al.* 1994; Uversky *et al.*, 1996; Silva *et al.*, 1992). In native proteins, however, the binding site is still considered to be basically the only site connected directly with function-related ligation. Hence, interference with protein function by ligands of nonbiological origin, of interest for medical applications, can be done essentially only in competition with the binding of natural ligands or by affecting the catalytic process itself in the case of enzymes (noncompetitive inhibition). This is the commonly accepted understanding of the mechanism of action of drugs and poisons. Consequently, only inhibition may finally be expected as the direct effect of attachment of foreign ligands to the binding site. In theoretical considerations, however, it is also possible to activate protein function by affecting natural ligation indirectly. Such an effect may be obtained particularly in respect to antibodies and receptors by arresting the biological ligand at the binding site of the protein, mimicking a naturally increased affinity in this way. Cooperative stabilisation of protein structure (Dill *et al.*, 1993; Freire *et al.*, 1992; Chan *et al.*, 1995; Xie & Freire, 1994) allows the production of such effects by fixation of ligand generated specific structural motifs even far from the binding site. In enzyme kinetics this mechanism would correspond to uncompetitive inhibition, which also means a decrease of the dissociation constant and an increase of protein-to-ligand affinity. It is rather unlikely, however, that nonbiologically designed compounds would noncovalently fix the structural motifs, which occasionally appear far from the binding site producing the activation effect required by pharmacology. Hence such an effect exists basically only in theoretical considerations.

SUPRAMOLECULAR LIGANDS – BINDING TO PROTEINS

Surprisingly, this activation effects with potential biological and medical uses, became more real recently after the finding that some dyes with rigid polyaromatic rings – elongated symmetric molecules which form supramolecular ribbon-like micellar entities (Attwood *et al.*, 1990; Skowronek *et al.*, 1995; 1998) fulfill the requirements. They represent compounds which may stabilize the protein conformations occasionally altered during functional activities. The question arises, what is unusual in these supramolecular compounds in water that allows them to bind to proteins outside the binding site as strongly as specific ligands and to compete with the natural intramolecular interaction that drives the destabilized protein conformation back to the initial structure? It is the apparently unique mechanism of complex formation by ribbon-like micellar ligand entities (Piekarska *et al.*, 1996; Stopa *et al.*, 1998; Skowronek *et al.*, 1998). It is essentially different from standard ligand-protein binding. The peculiarity of binding arises from the special supramolecular character of the ligands as well as from the sufficient accessibility of the protein for attachment of a large foreign entity (Cody *et al.*, 1999; Bahar & Jernigan, 1999). However,

not all self-assembling compounds exhibit such a complexation property. Only those that assemble into ribbon-like micellar bodies appear to meet the requirements (Stopa *et al.*, 1997; 1998). This group of ligands is represented particularly by bis-azo dyes – Congo red, Evans blue and others – which, in water solution, form the so-called chromonic mesophases (Attwood *et al.*, 1990) composed of ribbon-like micellar entities whose polymolecular fragments, integrated by strong self-assembling, may bind to proteins as single ligands (Piekarska *et al.*, 1996; Stopa *et al.*, 1997; 1998). The special binding activity of ribbon-like micellar ligands arises from (1) the large area of ligand–protein interaction contact (2) the possible shape-adaptability of noncovalently stabilized micellar bodies, and (3) the exposure of a significant hydrophobic portion of the molecules participating in the supramolecular organization (Stopa *et al.*, 1998), impossible to conceal in this particular shape of the micelle. If the self-assembling interaction is sufficiently strong and micellar entities form highly integral units as a result, they may adhere to many polymers, particularly those with periodic structural characteristics (Woodcock *et al.*, 1995). This appears to include proteins. The clefts or gaps within β-sheets which become available upon denaturation or are generated in native protein as a result of function-derived constraints, most probably represent receptor sites corresponding to these supramolecular dyes (Piekarska *et al.*, 1996). The periodicity of the β-peptide conformation and the hydrophobicity derived from the hydrocarbon amino-acid portions make the peptide chains and the ribbon-like micelle fragment fit each other, ensuring strong mutual binding (Fig. 1).

The key problem to solve in this case was to determine whether the studied dyes interact with protein as integral polymolecular units or simply as a collection of individual dye molecules organized as independent elements within the site of their attachment. The answer could come from studies comparing the self-assembling tendencies and the binding properties of different azo-dyes (Stopa *et al.*, 1997; 1998). Finding a correlation would indicate at least that supramolecularity is the dominating factor in the formation of adhesive protein ligands. For this purpose it was necessary to develop special tests permitting the evaluation of the integrity of supramolecular ligands and their binding to proteins (Stopa *et al.*, 1997; 1998). The self-assembling tendency, which finally determines micellar integrity, was assessed by measuring the effect of the resistance of a sieve bed to electrophoretic migration of ligand dyes after elimination of the charge effect by parallel reference to non-sieve systems. The difference between the rates of electrophoretic migration of a given dye (sample) in sieve (15% polyacrylamide gel) and non-sieve beds (glass beads), performed in the same buffer and pH conditions enabled the calculation of the sieve resistance coefficient and then the evaluation of the self-assembling tendency. This allowed a comparison of the stabilities of micellar entities formed by different anionic dyes.

The reliability of the method was independently verified by diffusion and dialysis. The complexation capability of the studied dyes was tested by their binding to heat-denatured and native protein molecules independently. Immunoglobulin G was chosen as the model protein for these measurements. The binding of dyes to heat-denatured (63°C, 20 min) human IgG was evaluated by simply measuring the number of dye molecules still bound to the protein molecule after removal of the excess dye on a Bio-Gel P-6 column. Many different dyes were found to bind to heat-aggregated IgG, and not particularly those whose self-assembling is efficient. However, the number of dye molecules bound per molecule of IgG was significantly higher in cases of strongly assembled dyes (Stopa *et al.*, 1998).

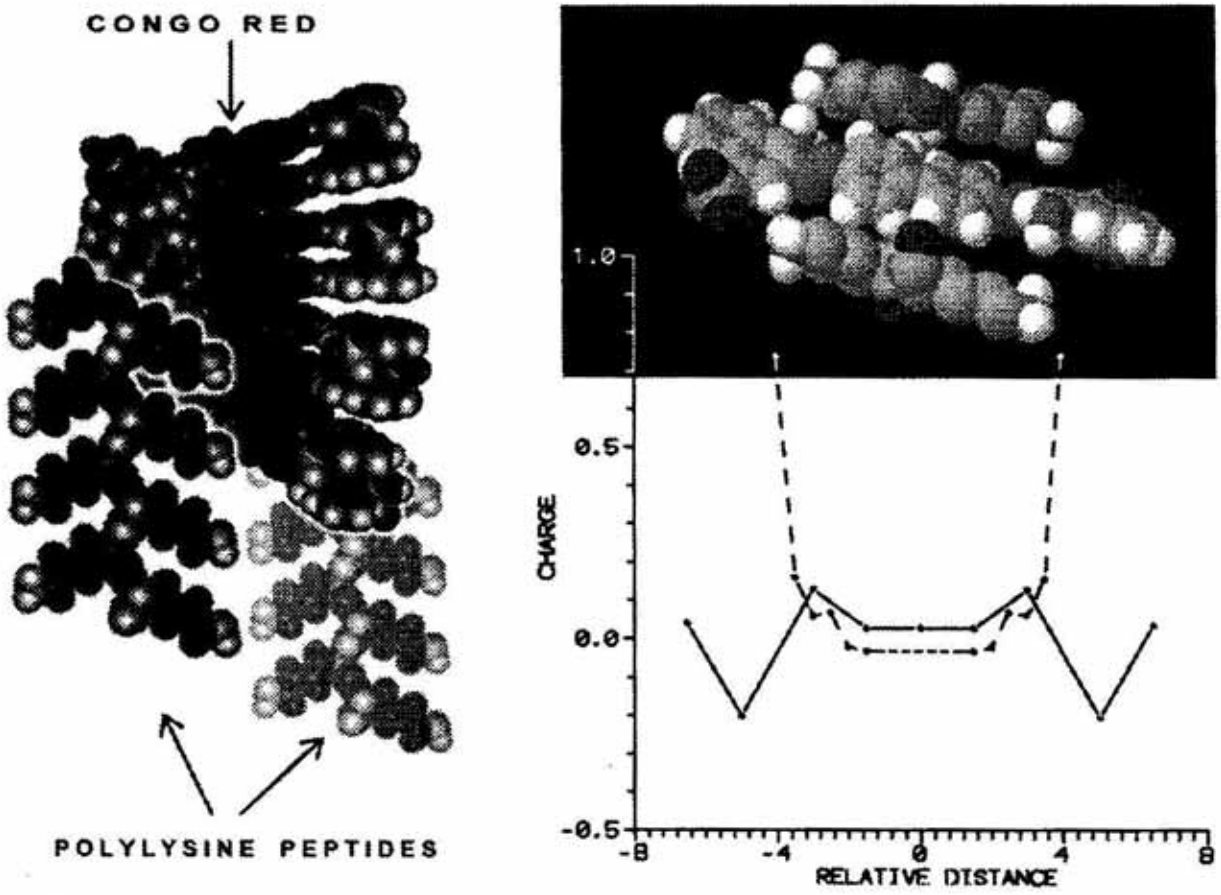

Figure 1. The structural correspondence of ribbon-like Congo red micelle and peptide chains of β-conformation (polylysine).

Shown are the model complex formation (left) and charge distribution along the complex as seen on the black field (right). Solid line – Congo red (total charge equals 0.0 ecu – charge distribution according to Resp procedure in Amber 4.1) (Kollman *et al.*, 1995). Dashed line – charged lysine dipeptide (charge distribution according to Parm94 data base in Amber 4.1) (Kollman *et al.*, 1995). Relative distance (arbitrary units) measured *versus* center of symmetry of the system.

CONGO RED BINDING TO ANTIBODIES

The attachment of the dyes to native immunoglobulin molecules (antibodies) was evaluated by direct measurement of the dyes attached to immune complexes, and the effect of enhancement on antigen binding (Stopa *et al.*, 1997; 1998). Antigen has been supposed to generate structural changes in antibody molecules (Roterman *et al.*, 1994; Lu & Schulten, 1999), which for years have been thought to play a role in transferring the signal triggering effector activity (Calvanico & Tomasi, 1979). In IgG, which is an all-β protein, these structural changes destabilizing the native domain conformation were assumed to create suitable sites for penetration and binding of supramolecular dyes. This assumption led to the idea of using self-assembling dyes to test the predicted selective binding of these compounds to antibodies in immune complexes (Rybarska *et al.*, 1991). The correctness of this hypothesis was confirmed experimentally. Immune complexes were proved to selectively bind Congo red by numerous assays, including isotopic labeling (Rybarska *et al.*, 1991), Ochterlony tests, fluorescence microscopy

analysis (Konieczny *et al.*, 1997), electrophoresis of soluble complexes (bis-TNP/anti-TNP system) (Rybarska *et al.*, 1995) and direct measurements of Congo red adsorption to SRBC/anti-SRBC complexes (Rybarska *et al.*, 1991). However, Congo red ligation to native antibodies was tested mostly indirectly by assessing the enhancement of antigen binding, which is an effect of simultaneous antigen and dye ligation. Predictions of the enhancement effect described by Rybarska *et al.* (1991) arose from the obvious assumption that the immunoglobulin conformation which is altered by antigen binding may be stabilized by simultaneous dye attachment, in consequence reinforcing antigen binding. The enhancement was measured by agglutination in the SRBC/anti-SRBC rabbit IgG system (Stopa *et al.*, 1997; 1998; Rybarska *et al.*, 1991). The experiments fully confirmed that only highly stable ribbon-like micellar bodies were able to evoke the enhancement effect (Stopa *et al.*, 1998). Poorly assembling or non-assembling dyes failed to enhance the immune complexation (Figs. 2 and 3.). Understanding this phenomenon helped to solve the problem of the contribution of direct antigen binding in immune signalization, which has been discussed for years.

The process of complement fixation has been known to depend on the gathering of antibodies by antigen, but it was also believed to depend on intramolecular structural alterations in the IgG molecule. The years-long debate has focused on the problem of the real role of the intramolecular structural changes generated by antigen binding (Zawodszky *et al.*, 1983) in the immune signaling, denied by some researchers (Metzger, 1990). The size, complexity and insolubility of immune complexes made it difficult to find experimentally convincing arguments to solve the problem, preventing a resolution until recently. What finally gave proof that antigen-binding-induced intramolecular structural alterations really do drive the immune signal was the use of highly assembled bis-azo dyes to stabilize

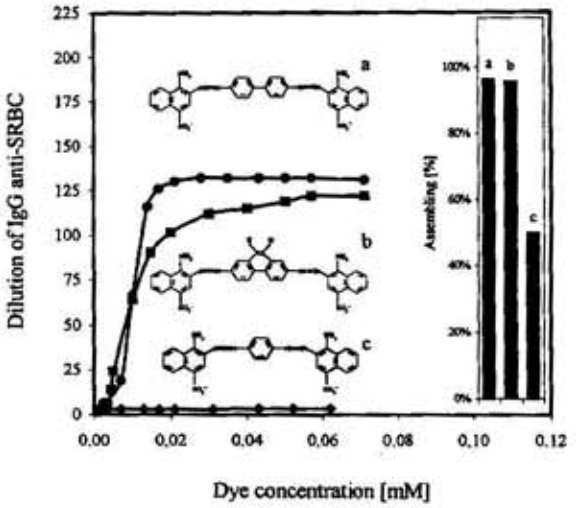

Figure 2. The enhancement effect of agglutination of strongly and poorly assembling dyes measured in the SRBC–antiSRBC system.

a, Congo red (●); b, 2,7-bis(1-amino-4-sulfonaphtyl-2-azo) fluorene (■); c, 1,4-bis(1-amino-4-sulfonaphtyl-2-azo)phenylene (◆). Black bars represent the assembling tendency of a dyes measured by electrophoresis in sieve and non-sieve beds. The enhancement of agglutination is expressed as the increase of maximal dilution of anti-SRBC IgG still yielding agglutination (Stopa *et al.*, 1997; 1998).

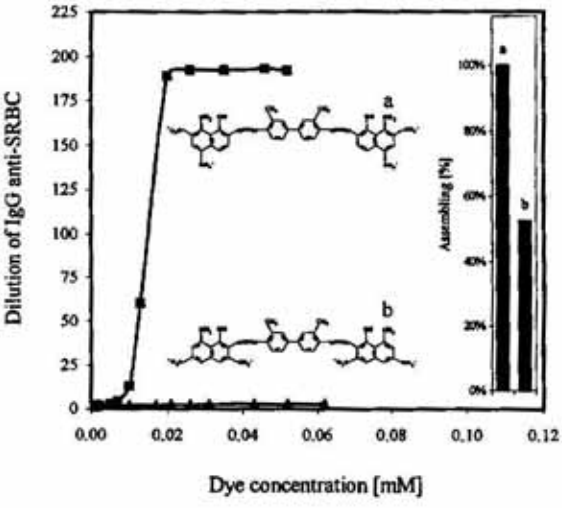

Figure 3. The enhancement effect of agglutination of highly and poorly assembling dyes measured in the SRBC–antiSRBC system.

a, Evans Blue (●); b, Trypan Blue (▲). Black bars represent the assembling tendency of dyes measured by electrophoresis in sieve and non-sieve beds. The enhancement of agglutination is expressed as the increase of maximal dilution of anti-SRBC IgG still yielding agglutination (Stopa *et al.*, 1997; 1998).

them (Rybarska *et al.*, 1991; Roterman *et al.*, 1993; Kaszuba *et al.*, 1993; Piekarska *et al.*, 1994). As predicted, Congo red was found to significantly enhance the antibody's affinity for the antigen. The enhancement was observed only when complete antibody molecules were used to form immune complexes. Neither Fab itself nor $(Fab)_2$ molecule interaction with antigen appeared enhanced by the influence of Congo red (Kaszuba *et al.*, 1993). The enhancement of antigen binding appeared very closely correlated with the inhibition of complement activation. Both effects increased simultaneously with an increase of Congo red concentration (Kaszuba *et al.*, 1993). This means that the supramolecular dyes really get in the way and interfere with signaling in immunoglobulins. Thus the concept of a direct functional conjunction between antigen binding and complement activation by intramolecular structural alterations finally has a convincing basis.

An independent insight confirming the easily induced low-energy conformational transformations in the IgG molecule came from studies of temperature-generated IgG susceptibility for dye binding (Piekarska *et al.*, 1988; Rybarska *et al.*, 1988). The classical two-state transition observed in thermodynamic analysis of protein chain unfolding basically registers the melting of the hydrophobic core. The structural changes in a still-native protein are highly reversible, and no significant population of intermediates is usually expected. However, since readily self-assembling dyes may form relatively stable complexes, penetrating to the peripheral parts of the protein molecule without necessarily being entrapped in the protein core, they seem useful to exhibit the local short-lived minima conformations induced by low temperatures (Piekarska *et al.*, 1996). Figure 4 shows that strongly self-assembling dyes start binding to the protein below its melting temperature, suggesting their possible correspondence to conformational changes induced by functional activation. The observed binding effect at

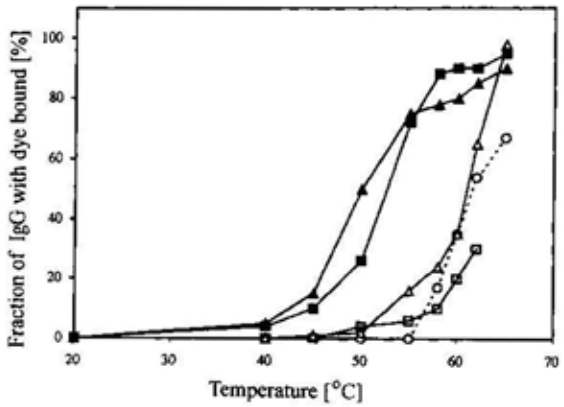

Figure 4. Heat-induced formation of protein (IgG) complexes with dyes of different self-assembling activity.

Congo red (■); 2,7-bis(1-amino-4-sulfonaphthyl-2-azo)fluorene (▲); Trypan Blue (Δ); 1,4-bis(1-amino-4-sulfonaphthyl-2-azo)phenylene (□). Dotted curve presents the standard heat-induced IgG aggregation registered as the formation of a high molecular mass fraction (Roterman *et al.*, 1994).

sub-denaturing temperatures (45–50°C), proving that the protein becomes accessible for complexation with the assembled dye far below the temperatures that initiate IgG aggregation, confirms that the assembled dyes attach to still-unaggregated individual protein molecules. On the other hand, even poorly assembled dyes were observed to bind to a molten protein core.

This indicates that intramolecular structural alterations rather than intermolecular organization cause the proteins to form receptor sites for supramolecular ligands.

The proposed mechanism of binding of bis-azo dyes to proteins suggests that different proteins containing β-pleated sheets may form receptor sites for supramolecular complexation of dyes.

BINDING OF CONGO RED BY SERPINS

Serpins are believed to belong to this group in particular, because of the naturally occurring unstable locus in the A β-pleated sheets, which allows various complexes to be formed

by serpins with proteases (Potempa *et al.*, 1994; Reif & Freitag, 1995) and also with micellar bodies of Congo red, as shown by computational modeling (Roterman *et al.*, 1998). A simple experimental procedure proved this to be true (Rybarska *et al.*, 1995). Two-directional agarose electrophoresis was used to investigate Congo red binding to proteins in the serum and in particular to serpins and serpin complexes (Fig. 5). Congo red was added to the second perpendicular electrophoretic run, allowing the migrating proteins to be met and overtaken by the dye molecules. Using the same conditions for both directions of electrophoretic migrations made all proteins, except for those bearing the bound dye, remain distributed along the diagonal line on the plate. Those with an extra charge from the bound anionic dye migrated faster and located above this line. Many protein complexes circulating in blood serum may be revealed in this way. All this confirms the predictions that supramolecularity may create ligands with nonstandard binding to proteins.

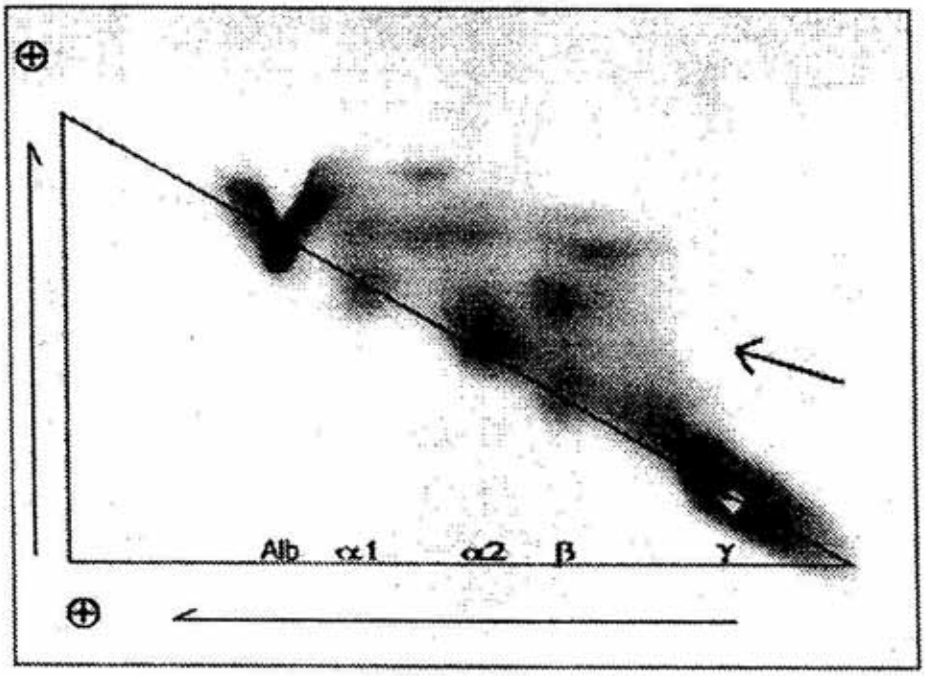

Figure 5. Acute phase serum protein complexes revealed by interaction with Congo red.

Congo red added to the second run in two-dimensional agarose gel electrophoresis meets proteins and increases their electrophoretic mobility. Arrow indicates extra charged Congo red–protein complexes located above the diagonal, where nonbinding proteins migrate (Rybarska *et al.*, 1995).

SINGLE MOLECULE AND SUPRAMOLECULAR FORMS OF CONGO RED IN ITS COMPLEXATION WITH PROTEINS – DISCUSSION

Congo red, which has long been used as a diagnostic stain for amyloids, was considered to be specific for this group of proteins (Glenner *et al.*, 1972; 1974; Klunk *et al.*, 1994; Pollack *et al.*, 1995; Safar *et al.*, 1994; Turnell & Finch, 1992; Carter & Chou, 1998). Different suggestions have been made to explain the mechanism of its binding; generally, monomer dye attachment to proteins is the explanation proposed (Ojala *et al.*, 1995; Klunk *et al.*, 1994; Glenner *et al.*, 1972; 1974; Carter & Chou 1998;). The location of individual molecules at the interface of two aggregated amyloid protein molecules of pig insulin, corresponding to this idea, was proved by X-ray crystallography data, indicating that such binding is possible (Turnell & Finch, 1992). However, the single dye molecule arrangement parallel to the peptide chain cannot be easily generalized to

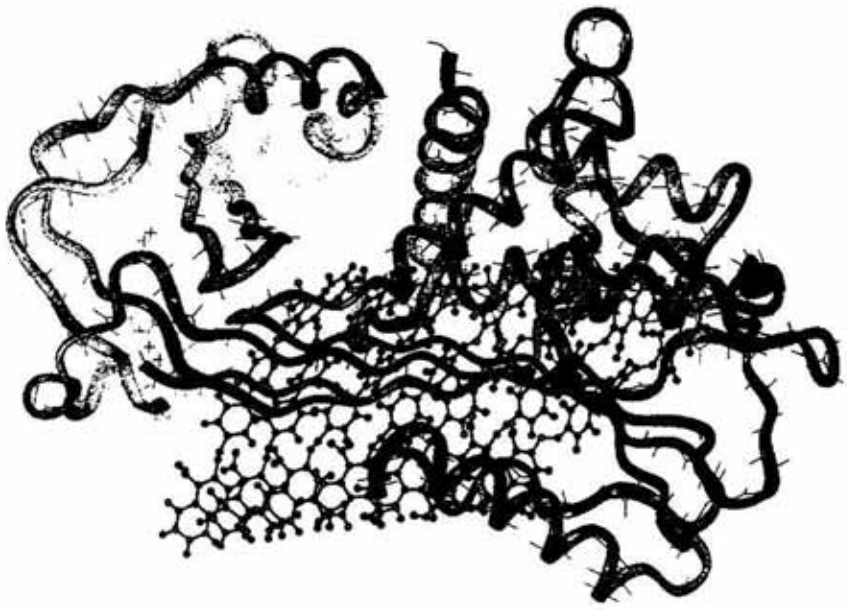

Figure 6. Serpin (9APP)–Congo red complex constructed by computation.

Ribbon, protein; balls and sticks, Congo red micelle (Roterman *et al.*, 1998).

larger proteins, since their long dye-binding clefts prevent the protrusion of highly polar sulfonic groups into the water environment.

Finding a specific site in a protein with positive charges and hydrophobic residues located properly for the given dye, seems unique and hence unexpected as a common event. The experimental studies and theoretical calculations indicated that the elongated, rigid, significantly nonpolar but still water-soluble Congo red molecules may be attached to proteins and peptide chains in different ways (Turnell & Finch, 1992; Roterman *et al.*, 1998; Skowronek *et al.*, 1998). The mechanisms of Congo red binding to the molten hydrophobic interior and to still-native molecules also differ. While both the self-assembling and non-assembling molecules may penetrate and be easily attached to the unstable (molten) protein core, the restrictions on penetration and binding of azo-dyes to native protein molecules appear very high (Stopa *et al.*, 1997; 1998). Only highly integral micellar fragments of dye may form sufficiently stable complexes with antibodies affecting antigen binding. This makes convincing the interpretation that supramolecular entities are required for the fixation of the easily reversible binding conformations that may become accessible in native molecules. The unique binding properties of such ligands and the perpendicular arrangement of dye molecules *versus* the peptide chain allows for dye-protein fitting with the best avoidance of steric hindrances and charges possibly conflicting with complexation (Fig. 6). These peculiar binding properties created by supramolecularity, which offer the possibility of interfering with functional activities of proteins, promise to be useful in experimental studies and medical applications.

Michael Jacobs helped edit the manuscript.

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