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THE EFFECT OF AZO DYES ON HEAT AGGREGATION OF IgG

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The mechanism of IgG heat aggregation was studied using IgG aggregates complexed with azo dyes to increase their solubility and stability. Heat dependent and heat independent steps of aggregation were differentiated. On heating IgG at the dye concentration exceeding 100 times that of protein, mainly dimers are formed, as judged from ultracentrifugation and chromatographic analysis, whereas high molecular weight derivatives appear at room temperature when the protein/dye ratio is decreased.

The analysis of spectral changes following either the attachment or removal of the dye from IgG aggregates implies that only a part of the dye molecules is bound firmly and directly to the protein binding sites. These dye molecules which are easily removed by adsorption to cellulose or reduced by dithionite but migrate together with IgG aggregates on chromatography and electrophoresis, are supposed to constitute that part of the micelle which extrudes from the binding site and, hence, is fixed indirectly to protein.

Various proteins with predominant β-structure were also found to bind azo dyes when heated.

Heat aggregation of immunoglobulins triggers their effector activity and the aggregates obtained are used for analysis of effector activities in immunosystems [1 - 4]. However, the mechanism of this activity still remains not clear.

In an attempt to elucidate this mechanism, soluble derivatives of heated IgG aggregates with azo dyes were formed. These derivatives differ in structure and reactivity depending on the azo dye used [5].

Since we have found that many other proteins interact with azo dyes

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during denaturation, the problem seems to be more general and attractive for theoretical reasons. The present paper extends previous studies on the structure of IgG aggregates.

MATERIAL AND METHODS

Reagents. β-Lactoglobulin and Concanavalin A were from Calbiochem, ovalbumin from Koch-Light. Dyes: Congo Red, Benzopurpurin 4B (POCh), Trypan Blue (Loba), Evans Blue (Serva) were used without further purification.

Isolation of protein-dye complexes. Human IgG, human transferrin and mouse IgG were isolated from pooled sera on cellulose DE-52 and further purified on Sephacryl S-300 column. The proteins at a concentration of 20 mg/ml were heated for 20 min at 62-65°C with a 100-fold molar excess of a dye. The high molecular weight protein-dye complexes formed were isolated by gel filtration on Sephadex G-200 sf or Sepharose CL-6B column (20×1.5 cm). The number of dye molecules bound to IgG was estimated spectrophotometrically: Congo Red at 505 nm, Benzopurpurin at 500 nm, Trypan Blue at 600 nm and Evans Blue at 630 nm using the absorption coefficients $A_{\text{cm}}^{1\%}$ of 46, 31, 52 and 58, respectively. In addition, IgG concentration was determined spectrophotometrically by subtracting the absorbance of dyes at 280 nm from the total absorbance of the sample. The absorption coefficient for IgG at 280 nm $A_{\text{cm}}^{1\%}$ is 1.4. All spectra were recorded using Unicam SP-800 spectrophotometer.

Sedimentation analysis. Analytical centrifugation was performed at 20°C in MON (Hungary) analytical ultracentrifuge fitted with Schlieren optics. Sedimentation velocity was determined at 30 000 rev./min at the concentration of IgG-dye complexes of about 3 mg/ml.

Digestion of IgG-dye complexes. Digestion was carried out in Tris/HCl buffer, pH 8.0, with trypsin (Sigma) at the enzyme/IgG ratio of 1:50, at room temperature. The reaction was stopped by addition of a threefold (w/w) excess of soybean trypsin inhibitor (Calbiochem). Thin layer gel filtration on Sephadex G-200 sf and polyacrylamide gel electrophoresis were used for the analysis of digests.

Reduction of Congo Red in the IgG-dye complexes. The Congo Red complexes with aggregated IgG (the dye/IgG molar ratio of 28:1) were reduced with sodium dithionate in 0.05 M Tris/HCl buffer, pH 8.0, containing 0.15 M NaCl, at room temperature, at 1:300 molar ratio.

RESULTS AND DISCUSSION

Formation and properties of dye-protein complexes

Moderately denaturing agents: heating (63°C, 20 min), urea (6 M, 24 h) or alkali (pH 11.6, 10 min) caused aggregation of human IgG with still
preserved large fragments of its monomer structure. As shown by Augener & Grey [6], Fab fragments are basically engaged in formation of aggregates while Fc remains unchanged [6]. The fact that heat aggregates precipitate readily, limits substantially their usefulness. However, the soluble form can be obtained, even at high protein concentration, if aggregation is conducted in the presence of azo dyes which form colloidal solution with rod-shaped micelles. Such a shape, predicted from packing restrictions, was confirmed by model studies and electron microscopy analysis (unpublished).

Immunoglobulin aggregates were usually complexed with Congo Red but other dyes were also used: Benzopurpurin, a methyl benzidine analogue of Congo Red, and two isomeric dyes: Trypan Blue and Evans Blue, with different substitution of sulphonic groups.

All these dyes react readily with the heat aggregating immunoglobulins but the extent of aggregation and the size of the aggregates formed are different for each dye (Plate 1, Table 1).

We have found previously [5] that interaction with protein affects the absorption spectrum of the dye, shifting its maximum towards the red. This indicates a relatively high binding capacity of azo dye and is in good agreement with the observed stability of the complex.
Table 1
Formation of IgG-dye complexes
For details see Methods

<table>
<thead>
<tr>
<th>Dye</th>
<th>Yield of aggregation (%)</th>
<th>( S_{20} ) of the main fraction</th>
<th>Dye/IgG molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congo Red</td>
<td>80</td>
<td>30-40</td>
<td>26</td>
</tr>
<tr>
<td>Benzopurpurin</td>
<td>73</td>
<td>30-40</td>
<td>26</td>
</tr>
<tr>
<td>Trypan Blue</td>
<td>74</td>
<td>60</td>
<td>4</td>
</tr>
<tr>
<td>Evans Blue</td>
<td>95</td>
<td>11.2 (dimer)</td>
<td>23</td>
</tr>
</tbody>
</table>

* The yield of aggregation without a dye was 37%.

Formation of the dye-protein complexes was recognized by the following criteria:
1. The same migration coefficients of a dye and protein on electrophoresis and gel filtration, under conditions in which migration of a free dye and protein is different.
2. The shift towards the red of the absorption spectrum of the dye complexed with protein.
3. Solubility of the complex under conditions in which proteins precipitate in the absence of the dye.

In general, the proteins containing large portions of peptide chains packed in \( \beta \)-sheet tend to form complexes upon denaturation in the presence of the dye. Among the proteins studied by us, immunoglobulins G (human, mouse), \( \beta \)-lactoglobulin, ovalbumin, Concanavalin A and transferrin reacted readily with azo dyes. Hair keratin was also classified as a dye-binding protein because of its conversion from the helical to \( \beta \) form on heating. Heating was a prerequisite since from the non-heated \( \alpha \)-keratin the dye could easily be removed by washing.

Some proteins interact with azo dyes without formation of aggregates (e.g. transferrin). Amyloid which for years has been known to bind Congo Red and stained with this dye for clinical diagnosis, was proved recently to contain a peptide chain folded in twisted \( \beta \) sheets [7, 8]. Surprisingly enough, subtilisin with peptide chain forming \( \beta \) sheets but showing a parallel configuration [9] failed to interact with the dye. Also proteins with predominantly helixed peptide chain (haemoglobin, cytochrome c, gelatine) did not bind the dye even on heating. In our other experiments we observed very poor binding of the dye by IgG denatured with propanol (25%, 20°C), i.e. under conditions when, according to Subramonia et al. [10], helixation rather than formation of \( \beta \) sheets prevails. SDS (0.01%, 20°C), due to stimulation of helix formation [11], caused complete dissociation of the dye from the dye-IgG complex.
The mechanism of dye binding and aggregation

The increase in absorbance and the shift in the absorption spectrum of the azo dye bound to protein is the most characteristic symptom of complex formation [5]. These changes may be due to the affinity of the binding site for the dye and/or to the number of dye molecules bound.

Concanavalin A is a good model for illustration of these effects because it binds Congo Red both in the native and denatured state but with different spectral effects. As can be seen from Fig. 1, heating increased the binding capacity of this protein with a simultaneous shift of the absorbance maximum of the dye.

![Absorbance vs Wavelength](image)

**Fig. 1.** Spectra of Congo Red complexes with native and heated Concanavalin A (concentrations of Concanavalin A and Congo Red 4.9 μM and 16.6 μM, respectively). 1. Free dye; 2. dye-Concanavalin A complex formed at room temperature; 3. dye-Concanavalin A complex formed on heating at 63°C for 20 min.

Usually, several molecules of the dye micelle are bound by a protein molecule [5]. It seems that only those dye molecules which are directly bound to protein, are responsible for the spectral effect. The remaining molecules of the dye micelle, although they migrate together with protein on electrophoresis and chromatography, behave like a free dye in solution and can be easily adsorbed by polysaccharides and reduced by dithionate (Fig. 2). It can be assumed that these molecules form the fragment of the micelle which protrudes from the protein.

Formation of large IgG aggregates seems to occur in two steps which may be separated and studied independently. At the dye/protein ratio of 100:1, IgG aggregates are represented mainly by dimers ($S_{20} = 11.2$) as shown by gel filtration on Sephadex G-200 using the azo dye (Congo Red) containing buffer. The appearance of dimeric forms of IgG was observed also by other authors studying IgG aggregation [12].
Large aggregates were obtained on decreasing the dye-protein ratio. This was usually performed on the Sephadex column, since Sephadex competes for the dye with migrating protein, provided buffer does not contain azo dye. However, even in such conditions dimers might be formed as in IgG-Evans Blue complexes since the latter dye has a higher affinity to protein than has Congo Red (unpublished). In contrast, in the complex with Trypan Blue the excess of the dye was easily removed at the very beginning of migration through the column and, consequently, only large aggregates appeared, as shown by sedimentation analysis (Table 1).

Heating is not necessary for formation of the large IgG-dye aggregates.

![Graphs](image)

**Fig. 2.** Kinetic differentiation of Congo Red molecules which directly and indirectly interact with aggregated IgG. A, Reduction of free (○) and bound (●) dye in the presence of a 300-fold excess of dithionate. B, Spectrum of the IgG-dye complexes, initial (—) and after 20 min of reduction with dithionate (—–). C, Shift in the absorption spectrum of Congo Red bound to aggregated IgG, visible on reduction of the complex with dithionate.

It seems, therefore, that denaturing conditions are only essential for unfolding of the peptide chain from its native β-conformation. Dimers are formed as a result of direct interaction of these unfolded peptides of heated IgG in the presence of the excess of azo dye, whereas large aggregates appear when the amount of the dye associated with dimers is decreased. This process may occur at room temperature and, hence, in conditions in which persistence of free unfolded peptide loops cannot be expected.
Fig. 3. Hypothetical mechanism of heat aggregation of IgG in the presence of azo dye.
1. Formation of dimers: A, Formation of a link of unfolded peptide chains between two
IgG molecules. B, Schematic presentation of five molecule fragment of the dye micelle
and the symbolic expression of the dye micelle (below). C, The dye-protein complex
(dimers). 2. Formation of large IgG aggregates. A, At the dye/protein ratio above 100:1,
formation of non-aggregating dimers. B, At low dye-protein ratio, the appearance of large
aggregates. C, Aggregates with the dye molecules limited to those directly interacting with
protein.

Fig. 4. Trypsinolysis of high molecular weight IgG-Congo Red aggregates isolated on
Sephadex G-200 (○), and the release of the dye measured as the decline of the \( A_{530}/A_{490} \)
ratio (●)
Thus, different mechanisms seem to be relevant for the heat-dependent dimer formation and dye-dependent association of dimeric species. The dye present in excess prevents formation of large aggregates since it might be bound to the same protein fragment which is responsible for stability of large aggregates.

Why the cross-bridging of heated immunoglobulin molecules stops at the level of dimeric forms, remains to be clarified. The schematic presentation of the proposed mechanism of IgG aggregation modulated by interaction with azo dyes is shown in Fig. 3.

The proposed role of cross-bridges in binding of azo dyes was supported by the results of short-term trypsin digestion of the complex [5]. The soluble IgG aggregates complexed with the dye are rapidly digested by trypsin. This indicates that, during partial proteolysis, the peptide chains of IgG participating in formation of intermolecular links, are primarily attacked. In contrast, the spectral changes of the complexed dye do not follow directly the desintegration of aggregates, thus proving that the dye remains in complex with products of digestion (Fig. 4). The proteolytic product associated with the dye was isolated by polyacrylamide gel electrophoresis under non-dissociating conditions and by gel filtration, and was found to be a peptide of $M_r$ about 30 000 [5]. The shifted towards the red absorption spectrum of the dye in this product proves that binding is of the same kind as in the nondigested aggregated IgG. This might imply that tryptic proteolysis cuts out the peptide fragment which forms the binding site for the dye in aggregates.

REFERENCES


