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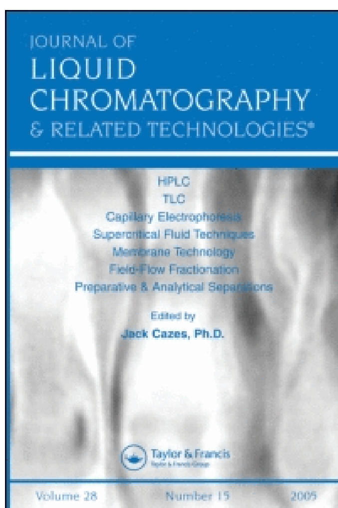

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Ligand-Induced Protein Transconformation Disclosed by Equilibrium Gel Filtration

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ABSTRACT

Multiple binding of some sulfonic dyes with trypsin in acidic media was investigated by equilibrium gel filtration. The apparent dissociation constants for the binding of flavianate, orange II, and orange G were, respectively, 46, 6, and 61 μM, reflecting differences in the organic moieties of the dyes. The elution profiles displayed a negative correlation of the eluted volumes between the monophasic trough and the peak with saturation ratios. This correlation was attributed to the outcome of distinct

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tautomeric forms of trypsin induced by ligand, together with the role of the dextran polymer and self-association of dye molecules. Protein transconformation was achieved after a lag time-depending on the ligand structure and concentration inside the column.

Key Words: Equilibrium gel filtration; Protein transconformation; Dye binding; Donnan equilibrium.

INTRODUCTION

Sulfonic dyes are known to interact with proteins leading to co-precipitates in crystalline forms.[1] Such crystals can be useful in protein isolation, dehydration, protection against denaturants, and crystallization studies.[2] The binding of organic ligands with proteins, was largely studied in the past by subtractive approaches of equilibrium dialysis using various methods for determining free ligand concentrations, like radioisotopes, complexing and extracting, partition equilibria between immiscible solvents, and spectroscopy.[3] Complexation of sulfonic dyes with proteins often results in co-crystal nucleation[4] that precludes the use of static equilibrium dialysis, due to bias on theoretical assumptions of Donnan equilibrium.[5] An alternative and dynamic method of equilibrium dialysis, developed by Hummel and Dreyer,[6] permits working with great ligand amounts without co-precipitating proteins.

In this technique, a Sephadex column is equilibrated with a ligand solution of low molecular weight, and the protein solubilized in the same solution is applied to the column. The equilibration between ligand and protein leads to a ligand depletion from a determined position in the column, resulting in a negative peak of the ligand, which left the column bound to the protein in the void. This equilibrium gel filtration method is considered more precise than static equilibrium approaches, as the free ligand concentration is not determined by analysis, but set at predetermined values.[3] The Hummel–Dreyer procedure is (a) faster than equilibrium dialysis,[7] (b) makes possible quantitative determination of binding constants in a single run,[8] (c) avoids corrections on binding calculations, due to unspecific adsorption of ligand that commonly occurs in equilibrium dialysis, (d) is able to be carried out with weak and unstable complexes,[9] (e) allows extracting thermodynamic constants for more than a single set of binding sites, with different affinities,[8] and (f) permits detection of time-dependent events of binding, by inspecting the position of the negative peak in the elution profile.

Time-dependent phenomena in enzyme kinetics were first suggested by Rabin,[10] while studying the thermodynamic possibility of co-operative effects in enzyme catalysis, due to substrate-induced conformational

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isomerization, that resulted in sigmoidal velocity–substrate relationships. The phenomenon of a slow transition in an enzymatic activity as response to rapid change in ligand concentration was named hysteresis.[11] The phenomenon has been extended to ligand–macromolecular interactions, other than enzymatic reactions, like helix–coil transition[12] and thermal relaxation of proteins.[13] In this work, we investigated the binding of some textile dyes to trypsin using the gel permeation equilibrium technique of Hummel–Dreyer.[6]

EXPERIMENTAL

Chemicals

Bovine trypsin (E.C. 3.4.4.4) was purchased from Sigma Chemical Co. (St. Louis, MO). The concentration of enzyme active centers was determined with *p*-nitrophenyl-*p*′-guanidine benzoate, according to Chase and Shaw.[14] The sulfonic dyes, flavianic acid, orange II, and orange G (Fig. 1) were obtained from Sigma Chemical Co. and further purified at the University of Minnesota, St. Paul. The concentration of purified dyes was determined spectrophotometrically using extinction coefficients previously determined.

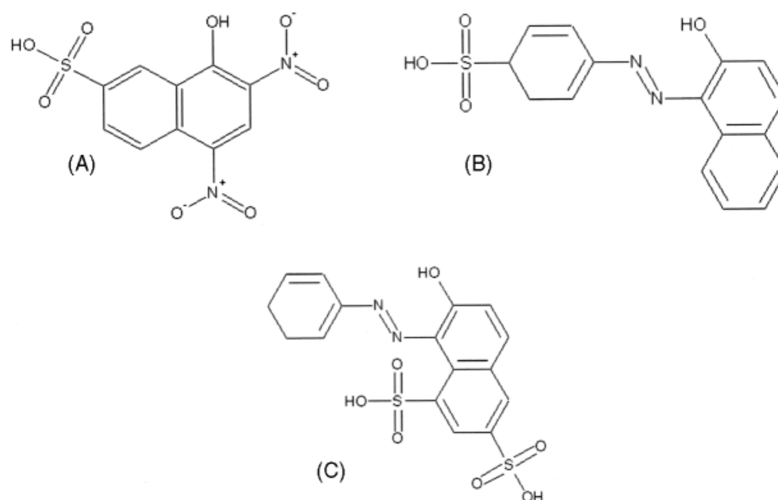

Figure 1. Organic moieties of the textile dyes used in the equilibrium gel filtration experiments. (A) Flavianic acid; (B) orange II; (C) orange G.

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Enzyme samples were prepared daily and centrifuged in a Sorvall instrument at 45,000*g* (SS-34 rotor) for 45 min, to remove a small insoluble residue. Other chemicals were of analytical grade.

Equilibrium Gel Filtration

The procedure was based on the method of Hummel and Dreyer,[6] as modified by Fairclough and Fruton.[15] Each column unit was developed in our lab and consisted of silica jacketed columns of $110 \times 0.4\,cm^2$, maintained at 22°C ± 1°C. The columns were packed with a deaerated slurry of Sephadex G-25 medium. After packing, columns were equilibrated with 10 volumes of 50 mM sodium citrate buffer, pH 3.0, containing the appropriate concentration of ligand ranging from 3 μM to 3 mM. The interaction of the dyes with trypsin was monitored, dissolving 5 mg of enzyme samples in 0.1 mL of each dye solution, at the same concentration of the equilibrium buffer and added to the top of the column. Flow rates were kept at 6.0 mL/hr exactly, during 24 hr with a Vera Monostat pump system (Sigma–Aldrich Inc., St. Louis, MO). The amount of dye bound to trypsin was obtained from the integrated areas of the trough (trapezoidal rule), derived from fraction volumes and corresponding optical densities, according to Eq. (1),[15]

$$PL = \frac{\sum(\Delta A \times \text{vol})}{\varepsilon} \quad (1)$$

where PL, moles of dye bound; ΔA, difference in absorbance between fraction i of the trough and the baseline; vol, volume of fraction i, in mL; ε, molar extinction coefficient of the dyes at the proper wavelength.

The trough volumes were used to avoid chromatographic effects due to binding of dye to protein that may occur when the positive peak is used. Control runs were carried out with dye solutions at the same concentration as those used with trypsin. The experiments were done in triplicate and the values were expressed as mean ± SD.

RESULTS AND DISCUSSION

Equilibrium Gel Filtration

Elution profiles can be illustrated in Figs. 2–4, [panels (A–C)] for flavianate, orange II, and orange G binding, respectively. The figures also present control runs with ligand baseline, dissociation constants, and peak-to-trough volumes for trypsin–dye interactions. Each profile exhibited a peak in ligand

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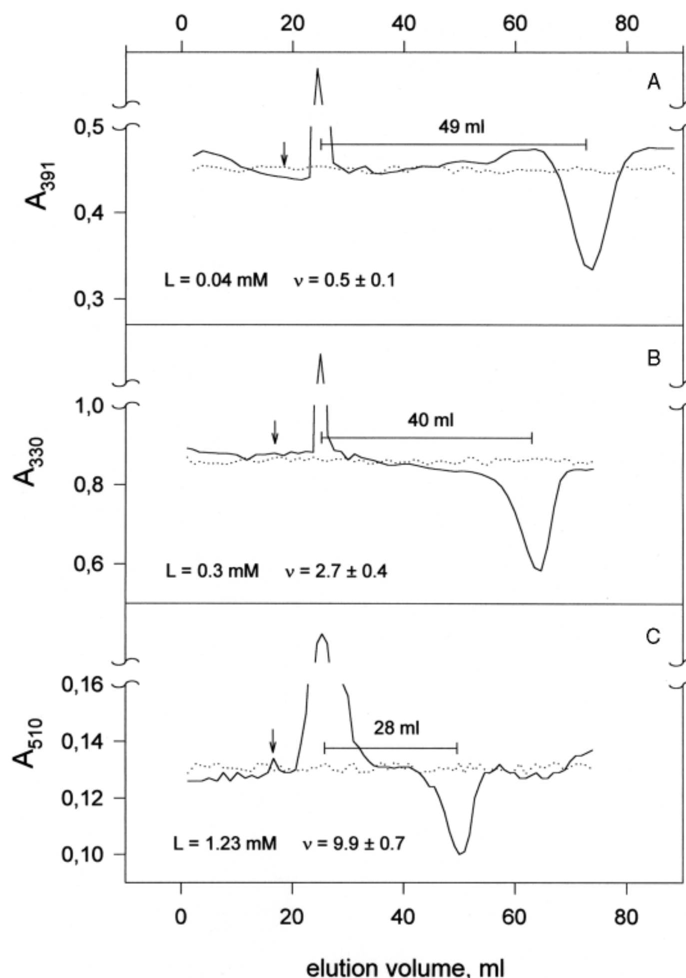

Figure 2. Elution profiles of equilibrium gel filtration for the interaction of trypsin with flavianate. The free molar concentrations of the dye were (A) 40 μM, (B) 300 μM, and (C) 1.23 mM. The arrows indicate the points where the trypsin solution was added. Dashed lines represent the elution of dye without protein.

concentration above the ligand–saturation baseline representing the excess ligand bound. A corresponding trough followed, representing the depletion that resulted from the bound ligand, which was carried ahead with trypsin.[15] The negative peaks shown in the gel permeation experiments (all panels of Figs. 2–4) were monophasic, ruling out heterogeneity of binding sites on

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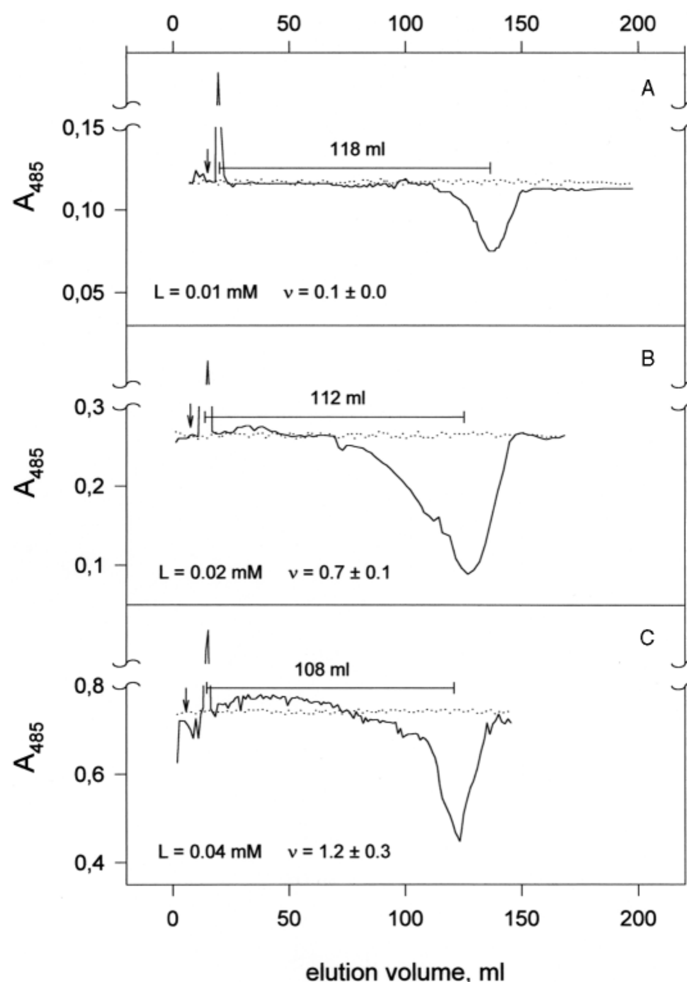

Figure 3. Elution profiles of equilibrium gel filtration for interaction of trypsin with orange II. The free molar concentrations of the dye were (A) 10 μM, (B) 20 μM, and (C) 40 μM. The arrows indicate the points where the trypsin solution was added. Dashed lines represent the elution of dye without protein.

the trypsin–dye interactions, as shown in the past, for a secondary binding site in trypsin bound with *bis*-benzamidine.[8]

The panels of Figs. 2–4 also show a broad shoulder following the peaks of bound ligand and before the troughs. This figure was related to systems when the ligand mediates association of macromolecules.[7,9] In this sense,

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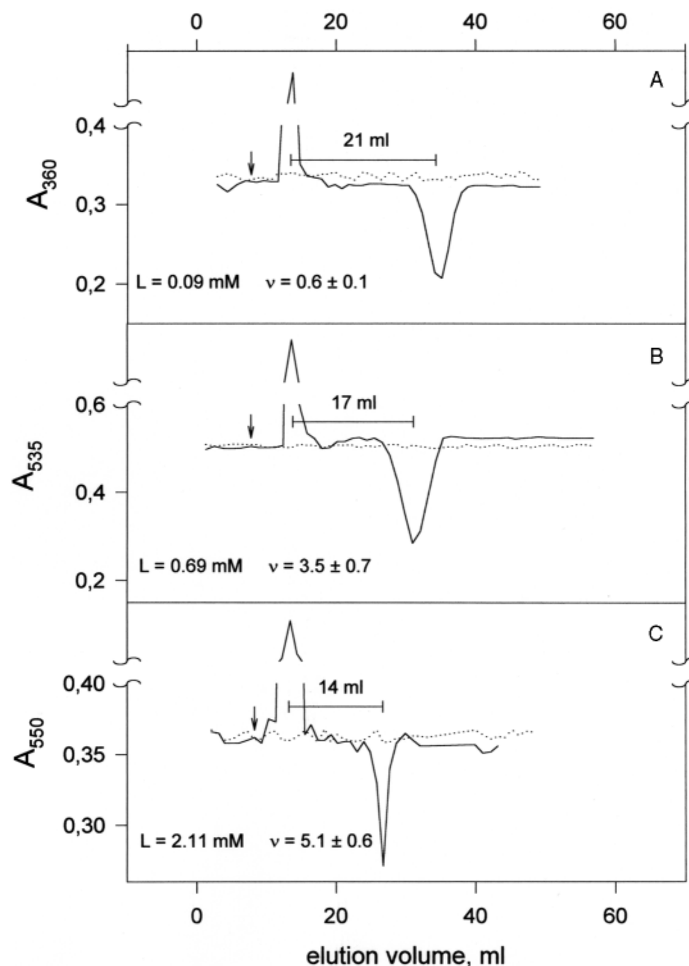

Figure 4. Elution profiles of equilibrium gel filtration for the interaction of trypsin with orange G. The free molar concentrations of the dye were (A) 90 μM, (B) 690 μM, and (C) 2.11 mM. The arrows indicate the points where the trypsin solution was added. Dashed lines represent the elution of dye without protein.

as the initial narrow band of macromolecule departs from the top of the column to begin its passage down the column in the exterior mobile phase, it removes ligand from the solution within the gel until the simultaneous ligand-binding and macromolecular association equilibria are satisfied with respect to the concentration of ligand added. At the same time, however,

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the concentration of macromolecule and its complexes decreases continuously as the band spreads due to axial dispersion. Consequently, macromolecular association is reversed by mass law action with concomitant release of ligand bound into the associated proteins.[16]

In brief, the dilution of trypsin inherent to the chromatographic procedure decreased the role of protein associations. This was remarkably observed with the binding of orange II to trypsin [Fig. 3, panels (B) and (C)].

Apparent dissociation constants for the interactions to the first enzyme site were calculated from the relations below,

$$PL = P + L \tag{2}$$

$$K_d = \frac{[P][L]}{[PL]} = \frac{([P_t] - [PL])[L]}{[PL]} \tag{3}$$

where $[P]$ and $[L]$, free molar concentration of protein and ligand, respectively; $[PL]$, molar concentration of bound ligand; $[P_t]$, molar concentration of total protein added; K_d, apparent dissociation constant of the complex.

Assuming that the number of sites filled by the dye at given conditions (ν) is determined by the molar ratio between the bound ligand and the total protein, one obtains:

$$K_d = [L]\frac{1-\nu}{\nu} \tag{4}$$

The resulting apparent K_d for the binding of flavianate, orange II, and orange G with trypsin were, respectively, 46 ± 7, 6 ± 1, and $61 \pm 5\,\mu$M. These values would reflect structural differences in the organic moieties of the dyes (Fig. 1). The major significant difference between flavianate and orange II is the presence of an additional aromatic ring in the latter. This added ring is known to contribute to an appreciable portion to the binding energy of dye–polymer complexes.[1] Another difference in the amount of Gibbs free energy, was also found between orange II and orange G, organic dyes structurally different in the number and location of their sulfonate groups (Fig. 1).

Binding studies carried out with BSA interacting with methylorange and azosulfathyazole[17] indicated that the additional sulfonate group of the latter does not participate in the binding, but remains free for interaction with the solvent water. This picture would be occurring with orange G decreasing the affinity for trypsin as compared with orange II. Furthermore, there is a high degree of coplanarity in hydrazo groups found on co-crystals of orange G with basic amino acids, which would allow for a great number of interactions between water molecules and the sulfonate groups of the dye.[18]

In addition, the two deactivating groups in flavianate and orange G, respectively nitro and sulfonate groups, could induce a positive character in

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the naphthalene rings, increasing the acidity of these dyes as compared to orange II. As a consequence, a higher eletrostatic repulsion in these dyes could lead to a decrease on its trypsin affinity. Finally, the electrostatic repulsion in orange G mediated by the two sulfonate anions, could counterbalance with the coulombic attraction on trypsin surface and hence compensate for the increase in affinity promoted by van der Walls attraction of the additional aromatic ring. The net result of the counteracting effects promoted by the additional ring and the additional charge could lead to an orange G–trypsin complex with a stability roughly the same as that of flavianate.

PROTEIN TRANSCONFORMATION

The elution profiles that resulted from equilibrium gel filtration also indicated a negative correlation of the eluted volume between peak and trough with the saturation range (Figs. 2–4, all panels). This correlation can be interpreted as a reduction on the binding affinity for the dye–protein complexes with increasing free dye concentrations.[8]

In the case of higher affinity, equilibration could take place close to the column top, leading to a greater separation between the protein peak eluted with the void and the ligand trough eluted later. A lesser affinity could result in equilibration at a lower location in the column and, as a consequence, a trough not as far from the protein peak, as can be shown in panel (C) of Figs. 2–4.[8] The negative peaks obtained, however, were monophasic, suggesting that the multiple equilibria of dye molecules with trypsin, proceeded with a single set of trypsin sites having similar affinities among them.[7,8] This strongly points to the possibility that distinct tautomeric species of dye–trypsin complexes, resulting from specific experimental conditions, could be interacting with free dye molecules. A similar result was found by Kumar et al.,[19] who observed a decrease of elution volumes in HPLC runs of bovine ribonuclease binding with increase trichloroacetic acid concentrations.

The affinity of the dye–trypsin complexes seems to be changed with the amount of free dye molecules, resulting in different chemical species of the macromolecule. The increase of dye concentration in the mobile phase of the column was surely involved in the increase of the interactions between the organic moiety of the dyes in solution and those interacting with the dextran structure of the Sephadex, thereby enhancing ligand polymerization. The self-association of dye molecules is thought to lead to a smaller chemical affinity of the free dye molecules, thereby limiting the formation of dye–protein complexes.[8] Parallel to that, the higher concentrations of the dye and trypsin colloid would have induced gel formation of the solution at

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the top of the column. This state of the colloid solution would decrease in magnitude throughout the column due to the solid matrix of Sephadex.

The combined effects of reduction, the chemical affinity for the dye and the gelification state of the colloid, therefore, could be the latecoming of a time-dependent event not observed in static equilibrium systems, due to the faster reaction velocity of the latter. Nonetheless, the soluble phase of dye–trypsin interactions, seems to lead to a structural stabilization of the macromolecule before a co-crystallization process in batch procedures.[20] This situation is reversed when dye–trypsin interactions overcome the free energy barrier of the non-specific interactions mentioned above. At this stage, trypsin suffers a massive transconformation that co-operatively enhance dye binding, resulting in the appearance of a trough at the top of the column [panels (B) and (C) of Figs. 2–4], a ligand-induced time-dependent event.

This two-step binding mode that results in protein transconformation has been observed with the binding of a second flavianate molecule to trypsin in dialysis equilibria experiments.[21] The increase on free dye concentration resulted in reduced peak-to-trough distances, as can be seem in the panels of Figs. 2–4. The largest peak-to-trough volumes for the orange II–trypsin complex would be attributed to its higher affinity, since the peak-to-trough resolution is proportional to the affinity of a particular ligand–macromolecular system.[8]

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CONCLUSIONS

The progressive denaturation of trypsin induced by some textile dyes seems to follow a ligand-dependence pattern in equilibrium gel filtration experiments, related to the elution volumes of the troughs with ligand concentration. This protein transconformation means that trypsin responds slowly to a rapid change in ligand concentration, resulting in a lag time in the response of the enzyme inside the column. This time could be explored in protein fractionation methods aiming at the determination of distinct dye–protein affinities, since the co-precipitation events seem to be preceeded by a time-dependence of protein transconformations, which can change with the dye–protein system used. This observation has significant consequences for the development of separation procedures based on protein–dye interactions.

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