Interaction of Poly (N-vinyl 2-pyrrolidone) with a Bisazo Dye in Aqueous Solution

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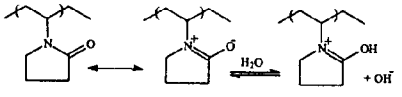
Summary: The binding mechanism of 6,6-[(3,3-dimethyl [1,1-biphenyl]-4,-4 diyl) bis(azo)] bis [4-amino-5-hydroxy-1,3-naphthalene disulfonic acid] tetra sodium salt, usually called Evan blue (EB), to poly (N-vinyl 2- pyrrolidone) (PVP) in aqueous solution has been studied spectrophotometrically at pH 9.2 in phosphate buffer in the presence and absence of NaCl. λ_{max} = 606 nm for EB solutions was observed and then by the addition of PVP red shifted to 636 nm. Analysis of results by Scatchard and Hill methods showed a mixed cooperativity mechanism at different [PVP]. Thus at high [PVP] there is positive cooperativity in the conversion of weak to strong complex and negative cooperativity at low [PVP]. The U-shaped Scatchard plot at fixed [PVP] and varying [dye] further supplements the operation of positive and negative cooperativity.

Introduction

Interactions in aqueous solution between small co-solutes with biopolymers such as serum albumin and synthetic macromolecules such as poly (vinyl alcohol), poly (vinyl imidazole), poly acrylamide, poly (methacrylic acid), poly (ethylene glycol) poly (N-isopropyl acryl amide) and some of their copolymers have been studied extensively [1-10]. Small co-solutes include organic dyes, drugs and toxic chemicals. Several methods exist for the analysis of protein and model interactions, each with distinct advantages and disadvantages [11-13]. Inter-action cloning methods are well suited for initial identification of binding partners and parameters but require secondary methods to verify complex formation and to determine the strength of interaction. More quantitative methods such as equilibrium dialysis [11,14], fluorescence, polarization and surface plasmon resonance [15,16] are useful for measuring binding affinities, but require purified and modified proteins and expensive equipment. Absorbance change method is easy in handling and commonly accepted to study the interaction. Takagishi *et al.* [17] examined the effect of temperature on the binding of pentyl orange to poly (N-vinyl 2 - pyrrolidone) (PVP), commenting on cooperative interaction and significance of hydrophobic interaction in the dye-polymer associations.



Structure 1: Possible tautomeric forms of repeating unit in PVP

PVP is amphiphilic in nature, having hydrophilic and hydrophobic moieties, so it might have different affinity toward different co-solutes (structure 1). After serum albumin, this polymer stand far above any of the other synthetic macromolecules to bind small molecules and displays strong binding affinity toward them when dissolved in water [18]. The binding of dye EB with model protein PVP has practical relevance because of its biological applications in blood volume determination and staining of reticuloendothelial systems [19]. The cooperative binding of ligand to protein can serve to increase their efficiency and regulate their activity, thus understanding of the mechanism of cooperativity/non-cooperativity is one of the central concerns of molecular biology. The azo linkage of the dye, which under certain specific conditions gives free amines, and use of amphiphilic PVP as blood plasma substitute and in pharmaceuticals add more weight to its importance for researchers. Attention is focused here on evaluating the contributions of ionic groups of azo dye, as well as the extent and strength of binding to PVP.

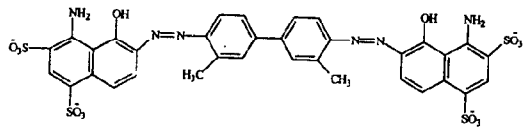
Results and Discussion

a): In the absence of salt

The present study was conducted at pH-9.2 with 1) fixed [dye] at various concentrations of PVP and 2) fixed [PVP] with various concentration of dye. In this section (a) only condition (1) applies. It has been asserted [8] that EB solutions obey the Beer-

Lambert law in the concentration range of 10 - 25 μmol dm^{-3}. Here the dye solution was scanned in triplicate over the whole range of wavelength, every time with fresh solution, but Beer-Lambert law was never obeyed above 6.5 μmol dm^{-3}, this is expected due to intense blue colour of the dye. So dye solution obeys the law at concentrations < 6.5 μmol dm^{-3}. Moreover at higher concentrations of dye solution, there is more probability of aggregation of dye molecules [22] due to adsorption or mechanical adherence on the wall of the cell as well as other glassware.

The absorption spectrum of the dye has λ_{max} = 606 nm and not 609 nm as reported elsewhere [8]. The spectral shift from λ_{max} = 606 to 636 nm (Fig-1) by addition of PVP quantifies the binding and this red shift motivated the selection of spectroscopy for the present study. An interconversion between different states of the dye molecule is the simplest explanation of the absorbance shift that occurs during binding. A similar shift was observed for alizarin red by addition of BSA [23]. This unusual spectral change of PVP-dye took place in two directions - first a decrease in absorbance at low [PVP] and then a shift in λ_{max} to higher wavelength with increase in absorbance at high [PVP]. At low [PVP] a small peak at 628 nm also appeared, which disappeared at high [PVP]. This indicates that at low concentration of polymer two species are present viz, the unbound dye and a weak PVP-EB complex, the latter converting to a strong EB complex on increasing [PVP]. This spectral change suggesting formation of different types of complex, which are probably due to interaction involving the phenolic hydroxyl group (ortho to the azo group) of the dye (strucrure-2), sulphonate anion of the dye with the cationic nitrogen and carbonyl group of the pyrrolidone ring (structure-1).



Structure 2: Structure of Evan Blue (EB)

Binding of the hydrophilic polymer to anionic dye is most likely to occur between the exposed hydrated negative oxygen end of the zwitterions and the hydroxyl group of the dye rather than between the latter and the positive nitrogen of the pyrrolidone, which possesses hydrophobic environment.

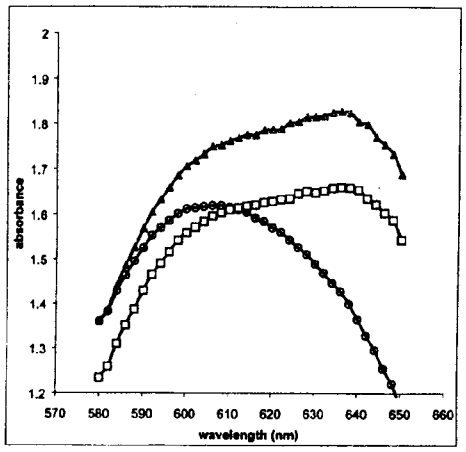


Fig.1: Absorption spectra of EB and PVP-EB complex at pH=9.2 (a)(O) = EB = 2.0μ mol dm^{-3}, (b)(□) = [PVP] = 2.0 μ mol dm^{-3} + [EB] 2.0μ mol dm^{-3}, (c)(Δ) = [PVP] = 10 μ mol dm^{-3} + [EB] 2.0μ mol dm^{-3}).

b): In the presence of salt

In this section (b) [dye] was 2.0 μmol dm^{-3}, several concentrations of PVP within the interval of 1-20μmol dm^{-3} were used and for each of these systems the concentration of salt in the aqueous medium was 0.2, 0.4 and 0.6M. The absorbance measurements were made at 636nm at 15 ± 0.2 ^{0}C and representative Scatchard plots are shown in Fig 2. The slope and intercept of these plots give the values of k and n for PVP + dye and PVP + dye + salts respectively, which are listed in table-1.

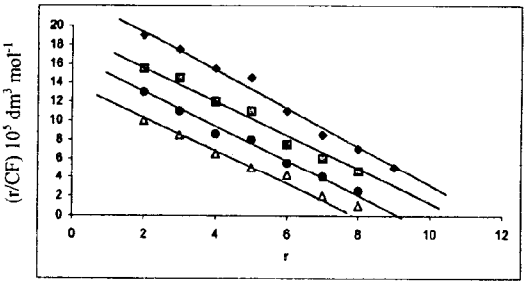


Fig.2: Scatchard plots for PVP-dye in the absence and presence of NaCl with pH 9.2.[PVP] = 1- 20μ mol dm^{-3} [EB] = 2.0μ mol dm^{-3}, (◆ = PVP + dye only), (□ = with 0.2M salt + PVP + EB), (● = with 0.4M salt + PVP + EB), (Δ= with 0.6Msalt + PVP +EB)

Table-1: Binding Parameters for PVP-dye and Related System.

System No.	System	N,	$K/10^3$ dm^4 mol^{-1}	References
S-1	PVP +EB	10.2	2.50	Present work
S-2	PVP + EB + 0.2M salt	9.66	2.06	"
S-3	PVP + EB + 0.4M salt	9.35	1.60	"
S-4	PVP + EB + 0.6M salt	8.33	1.50	"
S-5	PVP + TB	10.3	2.40	26
S-6	PVP + TB + 0.4M salt	7.57	1.03	26
S-7	HSA + EB + 0.12M salt	---	3.10	27
S-8	PVP + PND	24.6	0.46	14
S-9	PVP + TNS	1622	146	11
S-10	BSA + MO	22.4	0.49	25
S-11	BSA + Azosulfathiazole	--	1.25	25
S-12	PVP + MO	nk = 0.22x105		16
S-13	HB + EB +0.1M salt		3.1	29
S-14	BSA + HBP	536		19

Note: BSA = Bovine serum albumin, HSA = Human serum albumin, MO = Methyl-orange, PND=4-phenylazo-1-naphthol disulfonate, TB = Trypan Blue, TNS=2-p-toluidinonaphthalene-6-sulfonic acid, Salt = NaCl in S-2,3,4,6,7 and KCl in S -13. HBP = Hexadecyl pyridinium bromide

Different authors have reported the value of n and k in different units of concentration viz mole of dye /10^5 gram of polymer, number of sites/monomer unit, number of grams of dye/monomer unit and mole of dye/mole of polymer. Hence it is difficult to compare one system with another. We have recalculated all these values from their respective papers and unified them in to the same unit of the number of mole of dye/mole of the polymer for comparison with our system. This is the reason that an n and k value reported in table 1 may be different from the values in original papers.

Comparison among systems, S-1 - S-4 shows that both n and k decrease on incorporation of salt. This effect is explicable on the basis of denaturing i.e. breakage of hydrogen bonds by salt. Incorporation of salt at lower concentration produce a more pronounced binding reducing effect than occurs on increasing the salt concentration yet further. This small difference among the values lies in accord with viscometric findings [24] on the effect of guanidinium sulphate and sodium chloride with PVP. Values of n and k are similar for S-1 and S-5. This is reasonable since EB and TB are positional isomers. The same holds for 3 and 6 also. As far as comparison of S-2, S-7 and S-13 is concerned, many reasons are possible: a) PVP sometimes behaves differently from serum albumin. For example, Shikama and Klotz [25] found that methyl orange bound to serum albumin shows an absorption maximum near 435 nm, but the complex with PVP has a peak at 470 nm. The free dye in bulk water has a peak near 465 nm. Thus dye bound to PVP is in a much more water-like environment than that bound to serum albumin. b) This difference in behaviour may lie in the difference in conformation; PVP having an extended very open conformation, in contrast to the compact locally concentrated conformation of serum albumin. c) From the structural point of view EB should have higher k values than other azo dyes, because it has a greater number of groups capable of forming electrostatic interaction, hydrogen bonding, ion dipole interaction, hydrophobic bonding and even dipole dipole interaction. That is why S-1 has a higher k value than S-8, S-10, S-11 and S-12. d) Though the polymer is not true polyelectrolyte, evidence of this behaviour is not uncommon, especially in polymers having imidazole pendant group [28]. e) The last possible reason for variation of n is that some authors used the old Klotz equation instead of the modified Scatchard equation. In the Klotz equation the numerical value of the intercept 1/n is generally near zero and hence a small error in extrapolation gives greater uncertainty in the derived value of n. This uncertainty of variable n make it preferable to compare the binding affinity of compound on the basis of intrinsic binding constant k which can be evaluated more precisely without knowledge of n. The same holds for S-10 and S-12.

The high values of n and k, for S-9 are probably attributable to the fact that the authors used high molecular weight PVP sample (M.wt =360000), whereas in the rest the molecular weights are all 40,000 - 44,000.

In S-12, S-13 and S-14 the authors only gave the value of the product nk and of k respectively, which makes it impossible to calculate separate values of n and k in S-11, S-14 and the value of n in S-13.

c): Cooperativity / non-cooperativity

The value of the ratio [ligand]/[polymer] determines the cooperativity or non cooperativity in the binding studies, because when the magnitude of this ratio is very large, the number of available free sites per unit volume will be less than the free ligand concentration and vice versa. Meenakshi and Subramanian [26] observed cooperativity only at low value of the ratio [TB]/[PVP] i.e. a high [PVP] induced cooperativity whereas in a related system Reeves et al. [14] obtained it at a very much higher value of [ligand] / [PVP] ratio. In these systems the contents of dye and polymer were fixed and varied respectively. We adopted the reverse approach in the present study by fixing the [PVP] at 2.0 μ mol dm^{-3}

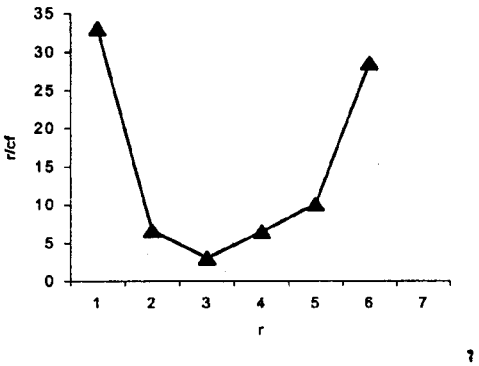


Fig. 3: Scatchard plot for PVP-EB system at pH-9.2. [PVP] = 2.0μ mol dm^{-3}, [EB] = 0.05 - 4.0 μ mol dm^{-3}

and varying the [dye]. The resultant Scatchard plot (Fig 3) is linear only up to low value of bound dye - polymer ratio, thereafter the plot is U shaped, which confirms [21] the existence of both positive and negative cooperativity.

Hill and Schwarz methods [30,31] have been used successfully as an alternative mode of analysis of cooperativity of different systems by different authors [4,27]. They are applied here also, as indicated below. The Hill equation is:

$$\log (r / n - r) = \log k_a + n_H \log C_F \qquad (5)$$

In equation (5) k_a is an apparent binding constant, n_H the Hill coefficient and n, already defined has an experimental value of 10.2 (table1). For the set of experiments in which the concentration of dye is fixed at 2.0 μ mol dm^{-3} and [PVP] is varied from 0.05 - 5. 0 μ mol dm^{-3}, it was found that 2.0 μmol dm^{-3} dye solution with [PVP] up to 0.2 μmol dm^{-3} has no change in the spectrum showing that 2.0 mole of dye can bind with 0.2 mole of PVP (10:1 mol / mol) without disturbing the nature of the complex. At [PVP] > 0.2 μmol dm^{-3} spectral change starts due to less polar environment around the already existing complex. Alternatively support is given to the proposed mechanism [26] that at low [PVP] every polymer chain has only one EB molecule bound to the site (through -OH group of the dye), the complex exist as a weak one which further promotes polar environment around the dye. On further addition of PVP, site-site interaction of the two neighbouring binding sites occurs and weak complex (λ_{max} = 628nm) is converted to a strong one (λ_{max} = 636nm). The collapse of weak complex (increase in absorbance which attains that of free dye) by the addition of salt indicates that hydrogen bonding is responsible mainly for the formation of weak complex.

The Hill plot (Fig 4) is not fully linear. The mid portion i.e. from 25 - 72% saturation is linear and shows cooperativity, whereas at very low and very high degrees of saturation, curvature indicating negative cooperativity [30] is evident.

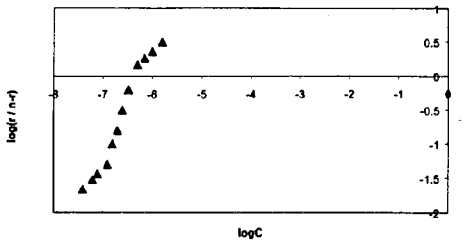


Fig.4: Hill plot for PVP-EB system at pH-9.2. [EB] =2.0 μmoldm^{-3} [PVP] = 0.05-5.0μmoldm^{-3}

When [dye] increases, the constructive interaction promoting positive cooperativity between two neighbouring binding sites diminishes and thus both the Hill and Scatchard methods give the same results. The non-applicability of the Hill equation at fixed [PVP] with varying [dye] further supports the mixed cooperativity.

Experimental

PVP K-40, having stated molecular weight 44,000 was purchased from BDH Chemical Co. Poole England. Evan blue (EB) and A.R NaCl were obtained from Aldrich Chemical Co. and Vickers Labs. Ltd. England respectively. The experiments were carried out in triplicate at pH 9.2 buffer using Hitachi Model U-2000 spectrophotometer with 1-cm cells. Care was taken to minimize the concentration loss by adsorption of the dyes with measuring glassware as well as cuvette of the spectrophotometer. The concentration range of the dye was 0.05 – 5 μmol dm^{-3} because above 6.5 μmol dm^{-3} it does not obey the Beer-Lambert law. For PVP the concentration range was 1 - 20 μmol dm^{-3}. For aqueous solution of dye alone the whole range of wavelength was scanned, affording λ_{max} of 606nm.

From the absorbance at λ = 606 nm for dye only and at λ = 636 nm for PVP - dye solution, the bound dye concentration C_B was calculated as follows [20]:

$$C_B = [(D - D_1) / (D_1 - D_2)] \times C_T \quad (1)$$

Where D_1 = the absorbance of free dye solution at 606nm

D = the partly bound PVP-dye condition i.e.at low [PVP]

D_2 = fully bound PVP-dye condition i.e. at maximum [PVP] and

C_T = the initial [dye].

The concentration of free or unbound dye (CF) is thus calculated as:

$$C_F = C_T - C_B \quad (2)$$

The ratio r of the concentration of bound dye to mole of PVP is:

$$r = C_B / [PVP] \quad (3)$$

In eq. (3) the concentration of polymer is in mole per dm^3 and r is calculated value that is related to the intrinsic binding constant k by the Scatchard equation, [21] which is based on a simple site-binding model:

$$r / C_F = nk - rk \quad (4)$$

In equation 4, n is the total number of binding sites per mole of polymer.

Conclusions

1: In aqueous solution Evan blue, a bis azo dye, has λ_{max} at 606 nm and a red shift to 636 nm occurs, when poly (N-vinyl-2-pyrrolidone) is added to it due to complex formation.

2: The weak complex formed initially produces a polar environment around the dye, thus promoting the formation of a strong complex.

3: There is a mixed cooperativity mechanism at different [PVP] concentrations; positive cooperativity in the conversion of weak to strong complex and in the strong complex at higher [PVP] and negative cooperativity at low [PVP] in the strong complex.

4: Inclusion of added NaCl reduces the polymer-dye interactions and thereby the value for intrinsic binding constant and the bound dye / polymer ratio derived via Scatchard plots.

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