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Binding of 1-anilinonaphthalene-8-sulphonate to poly(N-vinyl-2-pyrrolidone)

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Abstract. The binding of 1-anilinonaphthalene-8-sulphonate (ANS) to poly(N-vinyl-2-pyrrolidone) (PVP) of molecular weight grades k30 (molecular weight 40,000) and k90 (360,000) was studied by a dialysis technique in 0·05 M phosphate buffer, pH 7·1, at different temperatures. The intrinsic binding constant, K, was determined. The binding was favoured by negative enthalpy and positive entropy in both the systems indicating respectively that energetic forces and hydrophobic interactions were contributing to the binding affinity. The effects of addition of urea and palmitic acid on binding were investigated by dialysis and fluorescence techniques. The results showed that the binding of ANS to PVP was dependent on the nature and microenvironment of the binding sites and thereby pointed out the importance of the iceberg structure of water in the binding system.

Keywords. Binding; 1-anilinonaphthalene-8-sulphonate; poly(N-vinyl-2-pyrrolidone); dialysis; fluorescence.

1. Introduction

Poly(N-vinyl-2-pyrrolidone) (PVP) is a water-soluble synthetic polymer. It resembles the biopolymer bovine serum albumin in many respects, such as presence of amide linkage and preferential binding to anionic substrates. Owing to its excellent properties, PVP finds applications in many fields like the pharmaceutical industry, blood plasma substitutes, cosmetics and textiles. Because of its resemblance to biopolymers, the study of binding of ligands to PVP helps in understanding the functions of proteins and enzymes in physiological systems.

Ligands with a variety of structures, viz., dyestuffs (Frank *et al* 1957; Maruthamuthu and Sobhana 1979; Maruthamuthu and Dhandavel 1980; Maruthamuthu and Subramanian 1985), aromatic compounds (Molyneux and Frank 1961), drugs (Horn and Ditter 1982) and simple ions like fluoride (Maruthamuthu and Reddy 1984) have been reported to bind to PVP. In the present work we report the results of our study of the binding of 1-anilinonaphthalene-8-sulphonate (ANS), a hydrophobic and fluorescent probe, to PVP. Hydrophobic fluorescent probes are

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very useful (McClure and Edelman 1966) for locating hydrophobic binding sites in proteins and synthetic polymers; changes in fluorescence properties upon binding indicate conformational changes and binding site microenvironments of the macromolecules. An NMR study of the mechanism of ANS binding to PVP has already been reported (Kono *et al* 1973). Nevertheless a detailed study of the nature of binding, interacting forces and the effect of structuring of solvent medium on binding sites has not yet been made. Therefore the present study was attempted to investigate these factors to understand more about the ANS-PVP system.

2. Experimental

PVP of two molecular weight grades k30 and k90 (approximate molecular weights 40,000 and 360,000, respectively) was used. k30 PVP was obtained from Loba Chemie, India, and k90 from Sigma Chemical Company, St. Louis, USA. Water content of polymer samples was determined by drying a known quantity in an air-oven at 110°C for 2 days, and was found to be 13% and 6% for k30 and k90, respectively. Correction for this was made in expressing PVP concentrations. ANS was purchased as the free acid from Sigma, USA. Other chemicals used, viz., buffer reagents, urea and palmitic acid, were of reagent grade. All the chemicals were used without further purification.

All the experiments – dialysis, fluorescence and viscosity – were carried out in 0·05 M KH_2PO_4-Na_2HPO_4 buffer, pH 7·1.

The procedure for the dialysis experiment has been described elsewhere (Alexander and Block 1961). A concentration of $2{\cdot}25 \times 10^{-2}$ M in terms of monomer units was chosen for both the PVP samples. 10 ml polymer solution taken inside a semipermeable cellophane bag (Sigma, USA) was allowed to equilibrate against 10 ml ANS solution (in the concentration range 10–80 μM) for one day. A preliminary experiment showed that 16 h was the equilibration time and that binding of ANS to the cellophane bag was negligible. The free ANS concentration was determined by measuring absorption at 266 nm in a Carl-Zeiss UV-VIS spectrophotometer. The dialysis experiment was carried out at 3, 25 and 40°C.

The fluorescence emission of ANS was investigated at room temperature using an Aminco-Bowman spectrophotofluorimeter. The effect of urea and palmitic acid on binding was studied both in the fluorescence and dialysis experiments.

Viscosities of k30 PVP solution in buffer alone and in the presence of ANS were measured with an Ubbelohde suspended level viscometer at 30°C. The viscometer had a flow time of 134 s for double-distilled water and 137 s for buffer solution, which allowed us to neglect kinetic energy correction.

Linear regression analysis was used to calculate the binding parameters.

3. Results and discussion

The ANS-PVP system was analysed in terms of a simple model of binding. According to this, ligand molecules bind to linear independent binding sites on the

polymer. Based on this model, Klotz (Alexander and Block 1961) has proposed the following equation.

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{nKc}, \tag{1}$$

where r = number of moles of ligand bound per mole of monomer units of polymer, n = number of binding sites per mole of monomer units, $1/n$ = number of monomer units required to constitute one binding site, K = intrinsic binding constant and c = equilibrium or free ligand concentration. As required by (1), in a plot of $1/r$ vs. $1/c$, a straight line was obtained in each dialysis experiment (figure 1), proving that the above simple model of binding holds good in the ANS-PVP case also. From the slope and intercept, the binding parameters $1/n$, nK, the first binding constant, and K were calculated and are given in table 1. Since the experiment was made at different temperatures, thermodynamic parameters have been calculated using the temperature coefficient of K and presented in table 2.

The effect of urea and palmitic acid on the binding of ANS to PVP was studied using k30 PVP. The effect is illustrated in table 3 by the changes in r values. In dialysis experiments performed to study the effect of addition of these compounds, a lower PVP concentration ($3{\cdot}6\times10^{-3}$M) was used, whereas in the dialysis experiments earlier described a PVP concentration of $2{\cdot}25\times10^{-2}$ M was maintained. The reason for this has been described later.

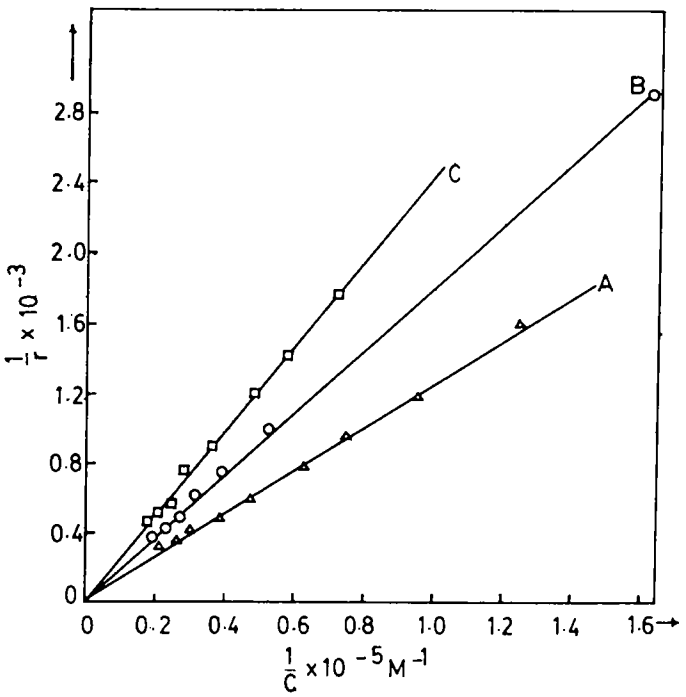


Figure 1. Klotz plot for the ANS-k30 PVP system. [PVP] = $2{\cdot}25\times10^{-2}$ M; 0·05 M buffer, pH 7·1. Determinations at A, 3°C; B, 25°C; C, 40°C.

Table 1. Binding parameters of the ANS-PVP system. [PVP] = $2{\cdot}25 \times 10^{-2}$ M; 0·05 M buffer pH 7·1.

	k30 PVP			k90 PVP		
Temperature (°C)	$\frac{1}{n}$	nK (M^{-1})	$K \times 10^{-3}$ (M^{-1})	$\frac{1}{n}$	nK (M^{-1})	$K \times 10^{-3}$ (M^{-1})
3		80·79	2·88[b]		149·63	13·11
25	35·63[a]	56·68	2·02	89·24	91·24	8·14
40		42·48	1·51		–	–

[a] Temperature coefficient on n is small. Hence a mean $1/n$ value has been given.
[b] K values have been calculated using mean n values.

Table 2. Thermodynamic parameters of the ANS-PVP system.

k30 PVP			k90 PVP		
ΔF^0 cal/mol	ΔH^0 cal/mol	ΔS^0 eu/mol	ΔF^0 cal/mol	ΔH^0 cal/mol	ΔS^0 eu/mol
−4507	−3050	4·89	−5332	−3968	4·58

Parameters were calculated using K values.

Table 3. Effect of urea and palmitic acid on binding. [k30 PVP] = $3{\cdot}6 \times 10^{-3}$ M; pH 7·1; Temperature 25°C.

	$r \times 10^3$		
$[ANS]_0$ (μM)	PVP + 2 M urea	PVP only	PVP + 20 μM palmitic acid
5	0·20	0·26	0·35
35	1·88	1·98	2·63
50	2·57	2·84	3·69
60	3·05	3·44	4·39
70	4·00	4·51	4·77
75	4·20	4·78	5·69

$[ANS]_0$ = initial ANS concentration.

Limiting viscosity numbers (intrinsic viscosities) of k30 PVP solution in buffer and in the presence of ANS of three different concentrations were measured from the intercept of a plot of reduced viscosity vs. concentration, using

$$[\eta]_{int} = [\eta_{red}]_{c \to 0} \cdot \tag{2}$$

Table 4 presents these values.

Figure 2 shows the fluorescence emission spectra of the ANS-k30 PVP complex in the presence and absence of the additives, the complex being excited at 370 nm. To minimise absorption (< 0·02) at the exciting wavelength, the concentrations of PVP and ANS were maintained as low as possible in the fluorescence experiments. For comparison, both fluorescence and dialysis experiments carried out to study the effect of additives used the same PVP concentration.

Table 4. Limiting viscosity numbers (intrinsic viscosities) of k30 PVP. Temperature 30°C; 0·05 M buffer, pH 7·1.

Sample	$[\eta]_{int}$ ml/g
PVP in buffer alone	21·22
PVP + 10 μM ANS	20·51
PVP + 40 μM ANS	21·58
PVP + 80 μM ANS	22·37

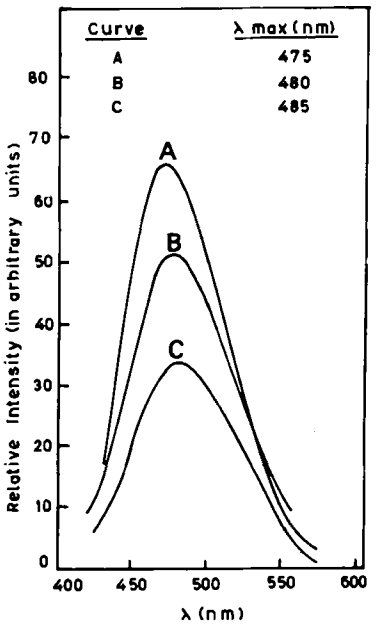


Figure 2. Fluorescence emission spectra of ANS-k30 PVP complex. Excitation wavelength = 370 nm; [PVP] = $3·6 \times 10^{-3}$ M; [ANS] = 10 μM; pH 7·1. A, PVP + ANS + 20 μM palmitic acid; B, PVP + ANS; C, PVP + ANS + 2 M urea.

Figure 3 displays the spectra of k30 PVP ($3·6 \times 10^{-4}$ M) in buffer solution (A) and in the presence of 20 μM ANS (B), the latter recorded using the same ANS solution as blank. In figure 4 curves A, B and C are spectra of ANS (20 μ M) in buffer, in $3·6 \times 10^{-4}$ M k30 PVP and in 9:1 (v:v) buffer-methanol mixture respectively. As before, to record the spectrum of ANS in presence of PVP, the same PVP solution was kept as reference.

3.1 *Affinity and interacting forces*

From the values of the intrinsic binding constants in table 1, we infer that both k30 and k90 PVP samples have moderately strong affinity towards ANS. This affinity, as evident from the data in table 2, comes from the large negative free energy

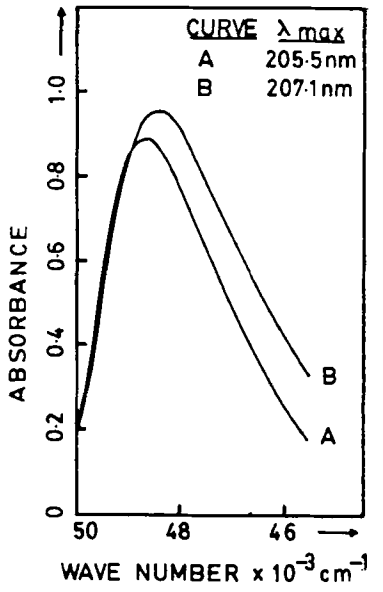


Figure 3. Spectra of k30 PVP in A, buffer solution; B, 20 μM ANS. [PVP] = $3{\cdot}6\times10^{-4}$ M; 0·05 M buffer, pH 7·1.

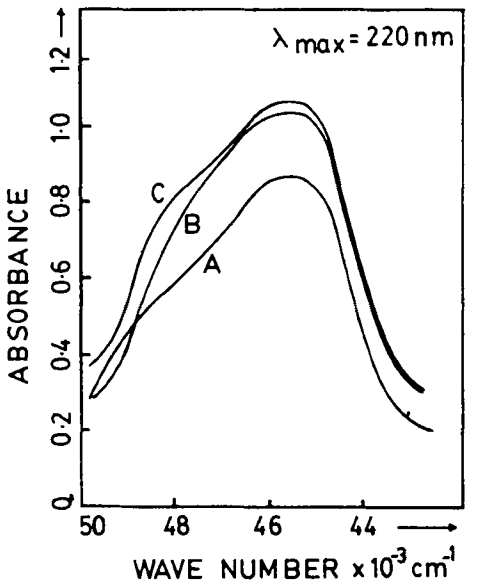
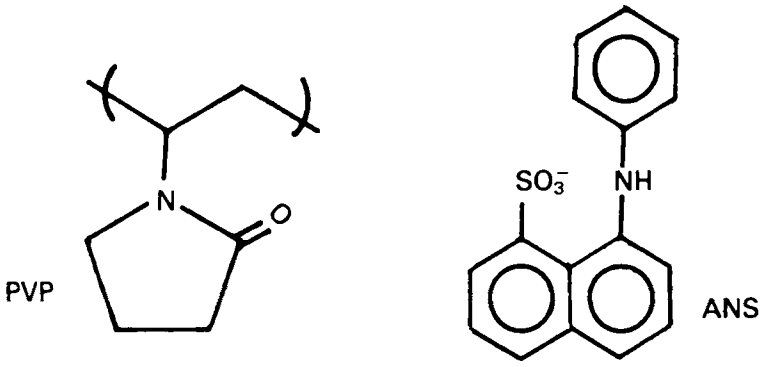


Figure 4. Spectra of ANS in A, buffer solution; B, $3{\cdot}6\times10^{-4}$ M k30 PVP; C 9 : 1 (v :.v) buffer-methanol mixture. [ANS] = 20 μM; 0·05 M buffer, pH 7·1.

change occurring in the binding process. Both negative enthalpy and positive entropy contribute to the observed free energy change. Thus the binding process is an energetically and entropically favoured one.

To explain the observed binding affinity, we have to consider the structural features of PVP and of ANS.



In PVP, the repeating unit is made up of two kinds of groups, i.e., the non-polar methylene and methine (CH) groups and the polar carbonyl group, with one hetero atom N at the centre of the pyrrolidone ring. The N and O atoms in the amide linkage carry fractional positive and negative charges respectively due to keto-enol tautomerism. The ligand molecule ANS is composed of non-polar aromatic rings with π electron cloud and one polar sulphonate group as the substituent. From the structures, the operation of the following forces is possible in the binding system.

(i) Electrostatic interaction of the ion-dipole type between the sulphonate group in ANS and the amide linkage in PVP.
(ii) Forces of dipole-induced dipole type between the carbonyl group in PVP and the easily polarisable aromatic rings in ANS.
(iii) Hydrophobic interaction between the aromatic rings in ANS and the non-polar groups in PVP.

The actual interacting forces which can be inferred from the experimental results are discussed below.

3.2 *Absence of ion-dipole forces*

Previous studies (McClure and Edelman 1966) on binding of 2-*p*-toluidinonaphthalene-6-sulphonate (TNS, structurally analogous to ANS) to bovine serum albumin have demonstrated that the probe binds to hydrophobic sites in the protein and that the sulphonate group does not play a significant role in binding. In TNS-PVP systems also (Takagishi *et al* 1978; Reeves *et al* 1981) the involvement of the sulphonate group in binding has not been invoked. Even the NMR investigation of the mechanism of binding of ANS to PVP (Kono *et al* 1973) did not reveal anything about the role of this group in binding. An obvious evidence for the non-involvement of this group in binding is as follows. Cross-linked PVP at neutral pH has been observed by us to have absolutely no binding affinity towards aromatic compounds with negatively charged groups, e.g., 1-naphthylacetic acid and naphthalene-1,5-disulphonic acid, whereas ANS, a similar molecule, does bind. This is possible only when the sulphonate group is not involved in binding. A similar situation is expected in the case of water-soluble PVP used in the present study. A possible reason for the non-involvement is the fact that the > NH and

sulphonate groups of ANS occupy the peri-positions of the naphthalene ring and have been shown to interact with each other (Balasubramaniyan 1966), thus preventing the sulphonate group from interacting with binding sites. As a result the operation of ion-dipole forces in the binding system may not be feasible.

3.3 *Dipole-induced dipole forces and spectral evidence*

The negative enthalpy change indicates the existence of energetic forces. But from the foregoing discussion, it seems that the forces of dipole-induced dipole nature originating from the polar carbonyl group in PVP and the easily polarisable π electron cloud in ANS are the predominantly contributing energetic forces. Spectral evidence, illustrated in figures 3 and 4, supports the above conclusion. The changes in the spectral properties of PVP and ANS on adding one component to the other clearly indicate the partial removal of water molecules and hence each provides with the other a less polar environment in the binding site. This point is proved by the spectrum of ANS in buffer-methanol mixture. The $n \rightarrow \pi^*$ transition of the carbonyl group in PVP is red-shifted with increase in intensity (figure 3). This shows that the group not only lies in a relatively less polar environment but also interacts with a non-polar group, i.e., the aromatic rings. The intensity of the $\pi \rightarrow \pi^*$ transition of ANS is enhanced (figure 4) when it binds to PVP. This enhanced intensity is possible only when the π electron cloud is perturbed and this happens when ANS interacts with the pyrrolidone rings of PVP.

3.4 *Hydrophobic interaction*

Hydrophobic interaction between two non-polar moieties is always associated with positive entropy (Nemethy 1967). Since the binding process in our system also is accompanied by positive entropy, it can be considered as a clear indication of the existence of hydrophobic interaction in the ANS-PVP system. The participating groups are the methylene and methine groups in PVP and the non-polar aromatic rings in ANS. This is supported by the observation that ANS shows fluorescence in k30 PVP solution ($3{\cdot}6 \times 10^{-3}$ M) but not in a solution of N-methylpyrrolidone, which is a monomer structurally similar to N-vinylpyrrolidone. PVP, because of its very open flexible conformational chain, is able to provide a non-polar environment whereas the N-methylpyrrolidone monomer solution of the same concentration containing only single entities is unable to do so.

3.5 *Affinity of k30 and k90 PVP's*

To get an idea about the effect of molecular weight on ligand binding, k30 and k90 polymer samples with the same monomer concentration were used. From table 1 we infer that k90 PVP has a 4-fold higher affinity to ANS than does k30 PVP. This can be explained by comparing the values of the thermodynamic parameters of the two systems. ANS-k90 PVP has a lower ΔH^0 value (table 2) but the entropy values of the two systems are more or less the same within the limits of experimental error. This shows that the higher affinity of k90 PVP is mainly due to the energetic forces, the hydrophobic interaction operating to the same extent. This inference can be explained by considering the $1/n$ values. One ANS molecule interacts with 89 monomer units in k90 PVP but only with 36 monomer units in k30 PVP. The

stronger interaction is responsible for the enhanced energetic forces in ANS-k90 PVP resulting in a lower ΔH^0 value. The similarity in contribution of hydrophobic interaction may be due to the similarity of the microenvironments of the binding sites in both the PVP samples. This is found to be true from the observation that in both the PVP solutions, ANS has the same fluorescence emission maximum (480 nm).

3.6 *Conformational change*

Viscosity can be exploited to detect conformational change in solute molecules. We have obtained 21·22 ml/g as the limiting viscosity number for k30 PVP in buffer at 30°C (table 4) which is quite in agreement with the value of 21·8 ml/g measured in water at 25°C (Molyneux and Vekavakayanondha 1986). Table 4 shows that there is only slight change in limiting viscosity number of PVP on addition of ANS at the concentrations employed. This implies that there is no significant conformational change in PVP on ANS binding and that the monomer units in the binding sites are not in rigid compact arrangement but are still flexible.

3.7 *Effect of additives*

Two additives, namely, urea and palmitic acid, have been employed in dialysis and fluorescence experiments, at concentrations 2 M and 20 μ M respectively. The very slight solubility of palmitic acid in water prevented us from using a higher concentration. The inferences obtained are interesting because they are helpful in obtaining some knowledge of the microenvironment of the binding sites.

Urea is a three-dimensional water structure breaker and thereby weakens hydrophobic interaction (Nemethy 1967) whereas palmitic acid [$CH_3(CH_2)_{14}COOH$] induces a hydrophobic hydration sheath around its molecular cavity because of its lengthy non-polar aliphatic chain. The latter is a more favourable condition for the strong hydrophobic interaction between PVP and ANS. Thus both urea and palmitic acid alter the microenvironment of the binding sites by affecting the iceberg structure of water. From their role, we expect that urea should inhibit ANS binding while palmitic acid should facilitate it. The observed experimental results support this expectation. The affinity for ANS is decreased in the presence of urea while it is increased in the presence of palmitic acid; this is clear from a comparison of the r values given in table 3. The binding of ANS by the fatty acid alone was tested by taking ANS in palmitic acid solution. But the very negligible intrinsic emission of ANS in buffer was not increased in the presence of palmitic acid. This shows clearly that ANS does not bind to the fatty acid and the increase in r value in the presence of the latter is wholly due to the enhanced binding of ANS to PVP.

The additives not only affect the amount of ANS bound by altering the microenvironment but also change the nature of the binding sites, as evident from the results of fluorescence experiments (figure 2). The ANS-k30 PVP complex has a fluorescence emission maximum at 480 nm. The addition of urea red-shifts it to 485 nm with reduction in intensity and increase in band width. But the palmitic acid blue-shifts it to 475 nm with decrease in band-width and increase in intensity. The emission maximum is very sensitive to the probe's environment. Its red-shift with band broadening indicates the existence of the probe in a relatively polar

environment while the blue-shift with band narrowing indicates a non-polar environment. Thus the binding sites on PVP become less non-polar in urea and more non-polar in palmitic acid solutions. This change in the nature of binding sites, as already discussed, is interconnected with the microenvironment, i.e., the ordering and disordering of water.

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