



A fluorescence analysis of ANS bound to Bovine Serum Albumin: Binding properties revisited.

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Introduction:

Determination of binding parameters such as the number of ligands and the respective binding constants need a considerable number of experiments to be performed. These involve accurate determination of either free and/or bound ligand concentration independent of the measurement technique applied. Then, an appropriate theoretical approach is used to fit the experimental data, and to extract the binding parameters. In this work, the interaction between bovine serum albumin (BSA) and 1-anilino-8-naphthalene sulphonate (ANS) is revisited using steady state fluorescence spectroscopy. The binding parameters for the ANS bound to BSA were determined and reviewed using Scatchard and Adair binding models with Klotz graph representation [1-3]. Halfman-Nishida approach was also employed for determination of free ligand concentration [4]. Job's plot and simulations were made in order to determine the scope and limitation for those methods. In addition, a new approach using the energy transfer from the tryptophan residues to the BSA-ANS complex is presented as a tool to help understand the binding mechanism of the albumin fluorescent complex.

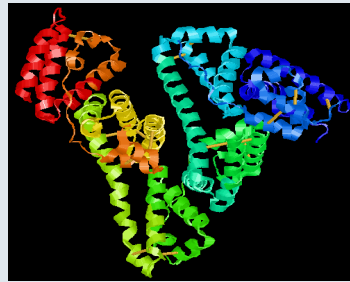
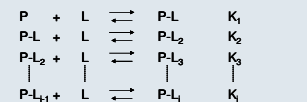


Fig. 1 – Serum Albumin Protein

Binding Equilibrium



$$[B] = [P-L] + 2[P-L_2] + 3[P-L_3] + \dots + (i-1)[P-L_{i-1}] + i[P-L_i]$$

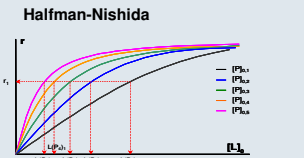
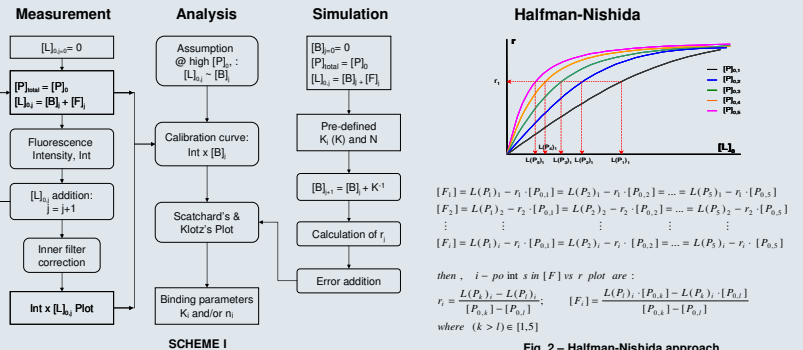
$$[P]_0 = [P] + [P-L] + [P-L_2] + [P-L_3] + \dots + [P-L_{i-1}] + [P-L_i]$$

$$[F] = [L]_0 - [B]$$

$$\begin{aligned}
 \text{Scatchard} \quad r &= \frac{\sum_{i=1}^N v_i K_i [F]}{1 + \sum_{i=1}^N K_i [F]} \\
 \text{Adair-Klotz} \quad r &= \frac{\sum_{i=1}^N i \times K_i \times [F]^i}{\sum_{i=0}^N K_i \times [F]^i}
 \end{aligned}$$

Experimental:

Bovine serum albumin, BSA (99%+ pure, Sigma), Phosphate buffered saline tablets for 0.01 M phosphate buffer (PBS) at pH 7.4 (Aldrich), and 1-Anilino-8-naphthalenesulfonic acid hemimagnesium salt hydrate, ANS (90%+ pure, Fluka) were used without further purification. All solutions were made up with purified water (Milli-Q Millipore). BSA and ANS concentrations for stock solutions were calculated using molar extinction coefficients $\epsilon_{280} = 43600 \text{ M}^{-1} \text{ cm}^{-1}$ [5] and $\epsilon_{350} = 5000 \text{ M}^{-1} \text{ cm}^{-1}$ [5], respectively. A Job's plot analysis [6] was used to determine the binding stoichiometry of the BSA-ANS fluorescence complex. In this approach, the molar fractions of ANS and BSA were varied while keeping the total concentration $([ANS] + [BSA])$ constant. The fluorescence intensity was measured and plotted versus the ANS molar fraction. For the free ligand concentration two methods were applied. First, a calibration curve related fluorescence intensity to total ANS concentration was built using high concentrated of BSA solution (10^{-4} M). Halfman-Nishida method (Fig. 2) was also employed to determine the free ANS concentration. Our approximation of the BSA structure in Scheme II was based on the crystal structure of HSA complexed with arachidonic acid obtained from the Protein Data Bank [28]. Scheme I summarizes the approach taken in this work.



$$\begin{aligned}
 [F]_1 &= L(P_1) - r_1 \cdot [P_{0,1}] = L(P_2) - r_1 \cdot [P_{0,2}] = \dots = L(P_5) - r_1 \cdot [P_{0,5}] \\
 [F]_2 &= L(P_1)_2 - r_2 \cdot [P_{0,1}] = L(P_2)_2 - r_2 \cdot [P_{0,2}] = \dots = L(P_5)_2 - r_2 \cdot [P_{0,5}] \\
 &\vdots \\
 [F]_5 &= L(P_1)_5 - r_5 \cdot [P_{0,1}] = L(P_2)_5 - r_5 \cdot [P_{0,2}] = \dots = L(P_5)_5 - r_5 \cdot [P_{0,5}]
 \end{aligned}$$

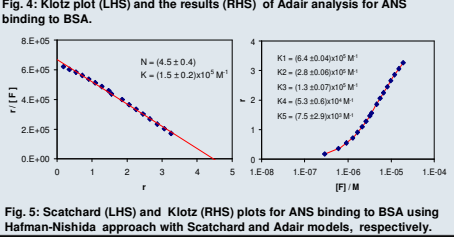
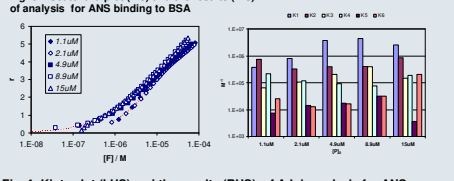
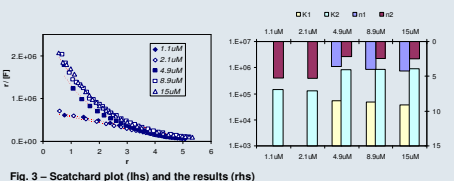
then, i -po int i in $[F]$ vs r plot are:

$$\begin{aligned}
 \frac{L(P_{0,i}) - L(P_{0,i})}{[P_{0,i}] - [P_{0,i}]} &= \frac{L(P_i) - [P_{0,i}] - L(P_{0,i})}{[P_{0,i}] - [P_{0,i}]} \\
 \text{where } (k > i) \in [1,5]
 \end{aligned}$$

Table 1 – Binding parameters recovered after simulating binding isotherm curves

Simulated	True	Adair model		Scatchard model	
		no error added	+10% error	without error	+10% error
K1	100	100.1 ± 0.3	151 ± 149	89.0 ± 0.3	144 ± 152
K2	10	9.94 ± 0.04	6.19 ± 6.85	9.930 ± 0.051	5.9 ± 8.0
K3	1	1.003 ± 0.004	0.51 ± 0.66	1.005 ± 0.005	0.38 ± 1.30
K4	0.1	0.0998 ± 0.0004	0.11 ± 0.14	0.099 ± 0.001	0.16 ± 0.53
K5	0.01	0.01014 ± 0.00003	0.004 ± 0.004	0.011 ± 0.000	0.004 ± 0.004

Results and Discussion:



The Scatchard model is relatively simple and thus it is the most popular binding model. However, analysis of the simulation data shows good fit curves, but that this yields erroneous binding parameters (Table 1). Therefore, this model may not represent correctly a ligand-protein system. The Adair (stoichiometric) model is a more general method (includes Scatchard case), but deals with the complexity of binding processes.

Method 1: Uses the linear part of the intensity vs. total ligand concentration.

A Critical Point: measuring the ligand free concentration: Bound ANS conc. is assumed to be directly proportional to fluorescence intensity. A calibration curve of the system at high protein conc. for the low conc. range of ANS must first be determined. In that linear region, it is assumed that ANS molecules are irreversibly bound to the protein.

A critical Problem: assuming that the binding constant is very high and equal for all the sites: In Fig. 3 and 4, the experimental data are graphically presented as Scatchard and Klotz plots. There is a clear difference between low (< 2.1 μM) and high (> 4.9 μM) BSA concentrations. At low protein conc., the calibration curve may underestimate bound ligand or, the binding sites have different affinities at low protein conc. For high protein conc., the curves are quite similar. **Scatchard analysis:** two sets of binding sites were observed for high protein concentration ($n_1 \sim 2$, $K_5 \sim 9 \times 10^4 \text{ M}^{-1}$; $n_2 \sim 4$, $K_2 \sim 5 \times 10^4 \text{ M}^{-1}$), and one class of binding site ($N \sim 5$, $K = 1.4 \times 10^5 \text{ M}^{-1}$) for low protein concentration. **Adair analysis:** 6 binding constant parameters were used to fit the experimental data (for high & low protein conc.). A general decrease of binding values are observed with the increase of occupation of binding sites ($K_1 > K_2 > K_3 > K_4 > K_5 > K_6$).

Method 2: The comparison of at least two binding curves with different acceptor concentrations can be used to obtain free ligand conc. Fig. 5 shows the results of binding isotherms analysis. **Scatchard model:** no distinction between the 5 binding sites affinities ($K \sim 10^5 \text{ M}^{-1}$). **Adair model:** five binding constants can be found, the number of binding sites range from 4-6.

Job plot Analysis: Another method to unravel the stoichiometry of a ligand-protein system is to build a Job plot (Fig. 6), and analysis of the plot shows that the intercept between the two slopes are at $x \sim 0.8$. The corresponding stoichiometry is 1:3.4. However, a curvature is also observed at the top of the curve is an indication that the total concentration (C_t) used is close to dissociation constant values for the binding sites [1].

FRET Analysis: The energy transfer (ET) efficiency between Tryptophan (Trp) and ANS at different binding sites can be used to correlate location with binding constants. The ET rate constant between the donor and acceptor, can be described as shown in scheme II. In a limited number of binding sites, the ET efficiency will depend on the binomial distribution of ANS bound around the donor centre. The probability of each random distribution will vary with the fraction of acceptor that is bound to the BSA with total number of binding sites. The probability function for the binomial distribution for P-L_i species, e.g., is the ratio of P-L_i and total concentration of BSA. Thus, the ET efficiency is related to the fraction of occupied binding sites allowed to correlate the average distance between binding site for ANS and Trp with the binding constant.

Fig. 7 five binding site case: with binding constant values determined by Adair-Hafman-Nishida method. The fitting show that the two highest affinity sites are located at distance greater than Forster radius, while the rest of low affinity sites are located at close to R_0 .

Trp-212 in BSA (Scheme II) is thought to be located in a hydrophobic micro-environment (sub-domain IIA), whereas Trp-134 is considered to be more exposed to solvent (sub-domain IA). It is at sub-domain IIA that most ligand is located, as determined by the arachidonic acid study. The active region where the Trp-212 can be used as a donor centre for ET involves almost all of subdomain-II, while the Trp-134 covers subdomain-I. The circles show the limited region for energy transfer of at least 50% of efficiency.

The majority of arachidonic molecules are located at a distance close to the Forster radius which agrees with our previous results that three binding sites are located at an average distance close to R_0 .

The structure also shows that subdomain III binding sites are partially or totally uncovered by the critical radius for Trp. Therefore, it may be expected that ANS molecules located at these sites will work less effectively as energy transfer acceptors.

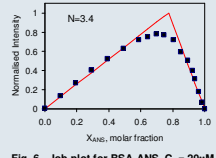


Fig. 6 - Job plot for BSA-ANS, $C_t = 20 \mu\text{M}$

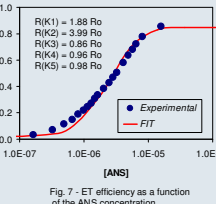
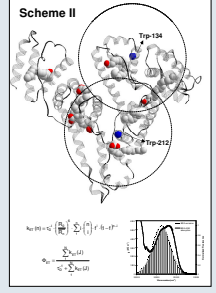


Fig. 7 - ET efficiency as a function of the ANS concentration.

Conclusions:

Using the Adair equation and Job's plot we find that ANS binds to bovine serum albumin at five different binding sites. Energy Transfer from tryptophan to bound ANS shows that the two highest associative sites are located away from the tryptophan residues (less efficient energy transfer) while three lowest affinity sites are found at, or near the Forster radius distance (24 Å). This agrees with our time-resolved fluorescence studies of BSA denaturation, two binding site types were found: one located towards the protein interior, the other more external and water accessible [1].

References

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