AN5: ANS-BSA complex formation.

Serum albumin (BSA) is a protein whose function in vivo is to transport several metabolites to different tissues. The study of ligand binding to it provides information about the functionality of this protein. 1-Anilino-8-Naphthalene Sulfonate (ANS) is a fluorescent probe that is extremely sensitive to the polarity of the solvent. It is practically not fluorescent in water (quantum yield 0.003), and its emission increases significantly in hydrophobic environments. When ANS binds to a hydrophobic patch of BSA the quantum yield increases significantly (from 0.003 to 0.65) [1].

ANS-BSA complex formation is well studied process that often used for testing of stopped-flow instrument performance. Fluorescence stopped-flow kinetics studies on the BSA--ANS interactions [2] show that the binding reaction is consistent with a two-step process. Double exponential kinetic of ANS-BSA binding was previously reported with correspondent second order association rate constant k^{+}= 3.5 x 10^{8} $M^{-1}s^{-1}$ for faster phase [3]

The ANS fluorescence decay is mono-exponential in homogeneous solutions with a lifetime ranging from 250 ps in water to 14 ns in ethanol [4].The fluorescence decays of ANS bound to BSA shows double-exponential kinetic with lifetimes about 2 ns and 16 ns [1]. Number of exponents in these experiments doesn't represent number of binding sites in BSA molecule that varies from 2 to 10 between reported studies [5-7]. Instead, it represents a number of groups of binding sites with different water accessibility. Binding sites that form ANS-BSA complex with short lifetime located on the molecule surface. They are accessible for water and ANS luminescence on them is quenched significantly. Binding sites inside BSA molecule are isolated from water and luminescence of ANS molecules on them has longer lifetime. The hypnotize about 2 classes of binding sites was proposed previously using data of flow injection gradient technique[8].

Dynamic of ANS binding to group of BSA sites with particular water accessibility was studied using double-kinetic technique. In order to maintain a pseudo-first order conditions ANS concentration was kept much lower than BSA concentration. Moreover in the excess of BSA it is reasonable to assume the only one ANS molecule is bound to BSA molecule and unnecessary complications with multi-molecular complexes can be avoided. Experiments were performed at T = 20C, in 50 mM MOPS buffer at pH 7.0. The solution of 0.1 uM ANS was mixed with BSA solution with concentrations 2.0 uM, 3.8 uM, 6.0 uM, and 8.2 uM. Concentration of BSA was determined spectrophotometrically at 280 nm using an extinction coefficient of 43600 M^{-1} cm^{-1} [5].

Third harmonic of laser (355 nm) was used for fluorescence excitation. Excitation intensity was adjusted by variable neutral density filter to keep maximum of signal in range 80-100 mV. Emission was collected through GG-420 cut-off glass filter and sheet polarizer oriented at magic angle. For each stopped-flow mix 500 signals were measured and then averaged in groups of 5 signals what reduced time resolution to 0.5 ms. All experimental data were averaged over 20 stopped-flow mixes.

The example of data is shown on Pic. 1. Measured fluorescence signals were fitted globally using 2-exponential decay function further addition of exponential terms did not improve the fit with both

lifetimes linked among signals. Lifetimes obtained from this analysis were in range 1.4-1.6 ns for short-lifetime component and 14.5-15 ns for long-lifetime component. Amplitudes of long-lifetime component are plotted as function of delay on Pic 2A. Double exponential curves provide excellent fit to all of data. Using linear fitting of observable rate constants for both, slow and fast phases, obtained in our experiment using equation $k_{obs}=k^{+}[BSA]+k^{-}$ we get k^{+} value of 144 $s^{-1}mM^{-1}$, what is in good agreement with conventional stopped-flow intensity measurements [3], and a previously unreported value of $k^{-}=824\ s^{-1}$. Amplitudes of short-lifetime component are plotted as function of delay on Pic 2B. They demonstrate single-exponential decay which rate constant are very close to the rate of the slow phase of the long life-time component.

The numeric simulation using a system of differential equations suggests this kinetic behavior could be a result of two competitive binding processes. ANS binding to water accessible BSA sites located near surface happens faster than it could be detected by used instrument. Binding to BSA sites located inside molecule is then controlled by concentration of remained free ANS during fast phase and then by process of ANS dissociation from binding sites on BSA surface due reversibility of binding process.

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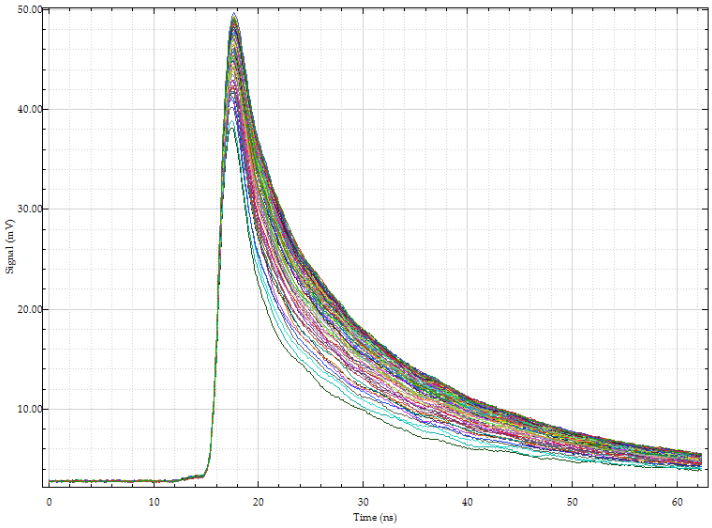
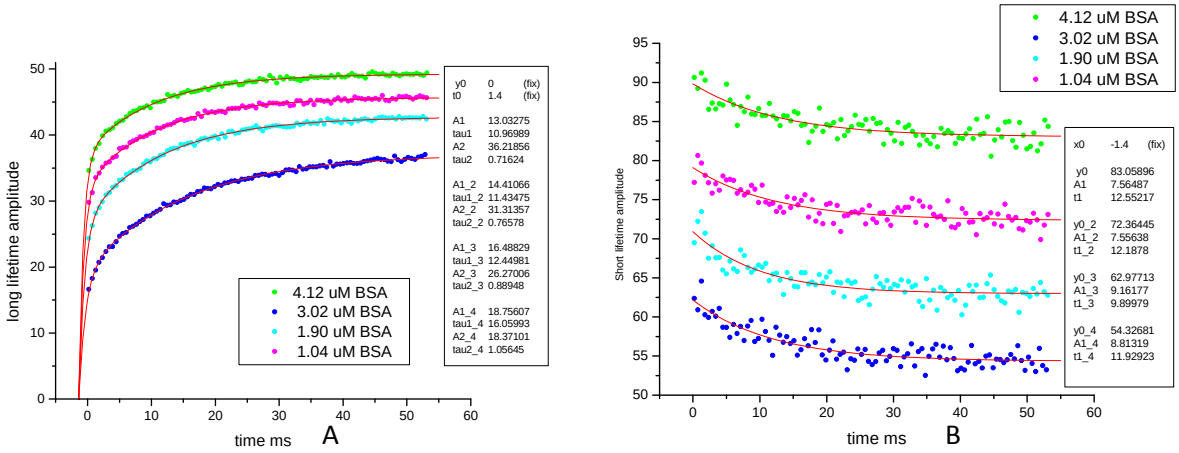


Pic. 1 Time-resolved fluorescence after mixing ANS and BSA in stopped-flow.



Pic. 2 Global analysis of ANS-BSA double-kinetic data. A: Amplitude of long-lifetime (15 ns) component vs time after mixing, for various BSA concentrations, best-fit to a 2-exponential model. B: Amplitude of short-lifetime (1.5 ns) component, fit to a single-exponential model.