KINETIC STUDIES ON BINDING OF BOVINE SERUM ALBUMIN WITH 1-ANILINO-8-NAPHTHALENE SULFONATE

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1. Introduction

There exist some dyes known as fluorescence probes which change their fluorescent properties upon binding with proteins [1,2]. The change in fluorescence intensity is sensitively dependent on kind and conformation of the proteins with which the probe interacts. One of the most representative systems of protein–fluorescence probe interactions is the binding of ANS (1-anilino-8-naphthalene sulfonic acid) to BSA (bovine serum albumin). Fluorescence intensity of ANS, which is very weak in aqueous solution, markedly increases when bound to BSA. Five ANS molecules are bound to one BSA molecule [3]. Although some evidence for microheterogeneity has been obtained from polarization of fluorescence of bound dyes [4], quenching of the protein fluorescence [4], and circular dichroism [5], fluorescence titration data suggest that the five binding sites of BSA may be considered roughly equivalent in binding affinity [3]. In spite of extensive studies by static methods, no kinetic investigation has as yet been carried out on this system. Kinetic studies so far made on the BSA–dye interaction seem to be confined to those using the temperature-jump or the pressure-jump method, by Froese et al. (azo dyes) [6], by Goldsack and Waern (Phenol Red) [7], and by Yasunaga et al. (Phenol Red, Methyl Orange, Bromo Cresol Purple) [8]. In this letter, we intended to study the kinetics of BSA–ANS interactions using the fluorescence stopped-flow method. The results are consistent with two-step mechanism including an unobservably rapid association process followed by a slow unimolecular process, both of them accompanied with the increase in fluorescence intensity.

2. Materials and methods

Crystalline BSA was purchased from Armour. Sodium salt of ANS was purchased from Tokyo Kasei Co. Ltd. and recrystallized as magnesium salt. The molar concentration of stock BSA solution was determined from the absorbance at 280 nm using the molecular weight of 66 000 and $\frac{1\%}{1\ cm} = 6.60$ [3]. The concentration of ANS was determined using the molar absorption coefficient at 350 nm of 4.95×10^3 [3]. Buffer solutions used were 0.1 M acetate (pH 5.4) and 0.1 M phosphate (pH 7.0).

Kinetic measurements were made at 25°C using a stopped-flow apparatus (Union Giken SF 70) with a cylindrical quartz cell of inner diameter 2 mm. The fluorescence emission was observed through a cut-off filter (transmitting above 430 nm) from the right angle to the excitation beam (360 nm). The dead time of the apparatus was about 1 msec under the operating conditions.

The reaction signals were stored digitally in a transient memory (Riken Denshi) and plotted on a section paper with an X-Y recorder (Riken Denshi F-3D).

3. Results and discussion

The interaction between ANS and BSA is of com-

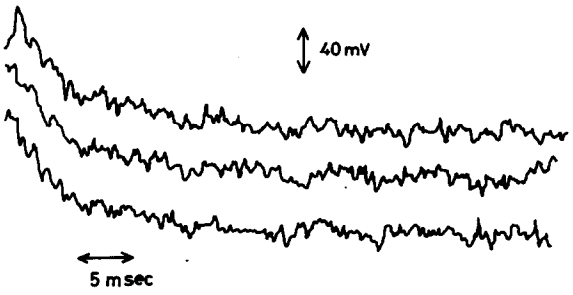

Fig. 1. The time course of fluorescence increase after mixing ANS and BSA at pH 7.0 and 25°C. The three curves are repeated runs under the same conditions. $A_0 = 0.566 \times 10^{-5}$ M, $B_0 = 4.74 \times 10^{-5}$ M.

plex nature in the sense that five ANS molecules can be bound per BSA molecule [3]. To avoid complexities where possible, the following experimental conditions were chosen with respect to the intital concentrations of BSA (designated B_0) and ANS (designated A_0).

Series 1 ($B_0/A_0 > 1$, fixed). In the excess of BSA, it may be reasonable to assume that only one ANS molecule is bound per BSA molecule. In this series, the relaxation time (the reciprocal apparent first-order rate constant) was measured with varied initial concentration of the reactants, the molar ratio B_0/A_0 being fixed at 4.2 and/or 8.4. Typical examples of the stopped-flow traces obtained for the increase in fluorescence intensity are shown in fig. 1. The time course

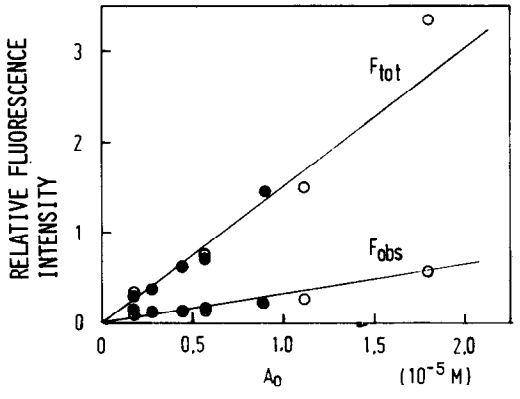

Fig. 2. Relative fluorescence intensity at $B_0 > A_0$ and pH 7.0, 25°C. F_{tot} and F_{obs} represent the static fluorescence increase and the change in fluorescence intensity observed with the stopped-flow apparatus respectively; •: B_0/A_0 = 8.4, ○: B_0/A_0 = 4.2.

followed the first-order kinetics giving a single relaxation time τ. However, the change in fluorescence intensity actually observed after the flow stops, F_{obs}, was appreciably smaller than the total change, F_{tot}, obtained in a static measurement. Fig. 2 shows the plot of F_{tot} and F_{obs} (both in volts) against A_0. The large discrepancy between F_{tot} and F_{obs} cannot be attributed solely to the dead-time of the apparatus (about 1 msec), but indicates that the reaction includes a process accompanied by the increase in fluorescence intensity which is too fast to be measured by the stopped-flow method. The proportionality between F_{tot} and A_0 suggests that ANS present is almost completely bound with BSA, i.e., the fraction of free ANS can be ignored. Fig. 3 shows the plot of $1/\tau$ against the sum of the equilibrium concentrations of free reactants, which is well approximated to $(B_0 - A_0)$, under the condition employed. This plot obviously shows the 'saturation', indicating that the reaction is not a single bimolecular association process for which $1/\tau$ should increase linearly with $(B_0 - A_0)$ [9]. This concentration dependency of $1/\tau$ bears a typical feature expected from a two-step mechanism involving a fast bimolecular association followed by a slow unimolecular process [9],

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AB \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} AB^* \qquad (1)$$

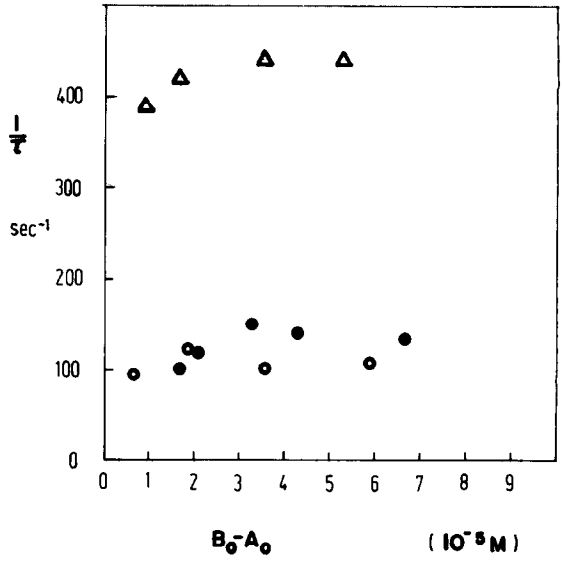

Fig. 3. Plot of $1/\tau$ vs. $(B_0 - A_0)$. •: pH 7.0, B_0/A_0 = 8.4; ○: pH 7.0, B_0/A_0 = 4.2; △: pH 5.2, B_0/A_0 = 8.4.

where k's are the rate constants of the steps specified above. The two relaxation times τ_1 and τ_2 are then given by Eqs. 2 and 3 [6]:

$$1/\tau_1 = k_1 (B_0 - A_0) + k_{-1} \quad (2)$$

$$1/\tau_2 = \frac{k_2 (B_0 - A_0)}{k_{-1}/k_1 + (B_0 - A_0)} + k_{-2}. \quad (3)$$

The results obtained (fig. 3) are consistent with the interpretation that only the slower process has been observed under the saturation level ($k_{-1}/k_1 \ll (B_0 - A_0)$). This is also in agreement with the F_{obs}/F_{tot} ratio stated above.

Series II (A_0 fixed, B_0 varied) In this case, the binding of more than one ANS molecule per BSA molecule must be considered, which would certainly lead to complicated rate equations. In this paper, we confine our attention only to an extremely simplified case, assuming that n (=1∿5) molecules of ANS are simultaneously bound to n out of five identical binding sites of BSA in a two-step mechanism analogous to Eq. 1. Then the scheme may be written as follows:

$$nA + B \underset{k^n_{-1}}{\overset{k^n_1}{\rightleftharpoons}} A_nB \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} A_nB^* \quad (4)$$

where A_nB and A_nB^* are the intermediate and final complexes between n molecules of ANS and BSA respectively, k^n_1 and k^n_{-1} are forward and reverse rate constants for the first process, and k_2, k_{-2} are unimolecular rate constants for the isomerization of ANS–BSA complexes.

When the second step is much slower than the first, the reciprocal relaxation time observed for the slower relaxation process, τ_2, is given by

$$1/\tau_2 = \frac{k_2 (\bar{A} + \bar{B})^n}{k^n_{-1}/k^n_1 + (\bar{A} + \bar{B})^n} + k_{-2} \quad (5)$$

where $\bar{A}$ and $\bar{B}$ are the equilibrium concentrations of ANS and BSA, respectively. When the equilibrium is very far to the binding side, Eq. 5 reduces to simple forms depending on the relative magnitude of A_0 and B_0:

a) $A_0/B_0 < 1$; Eq. 5 reduces to Eq. 3, i.e., n=1, $\bar{A}$=0, $\bar{B}$=$A_0 - B_0$.

b) $1 < A_0/B_0 < 5$; So far as $\bar{A}$=$\bar{B}$=0, Eq. 5 is simplified into

$$1/\tau_2 = k_{-2}. \quad (6)$$

c) $A_0/B_0 >$; all of the binding sites of BSA are

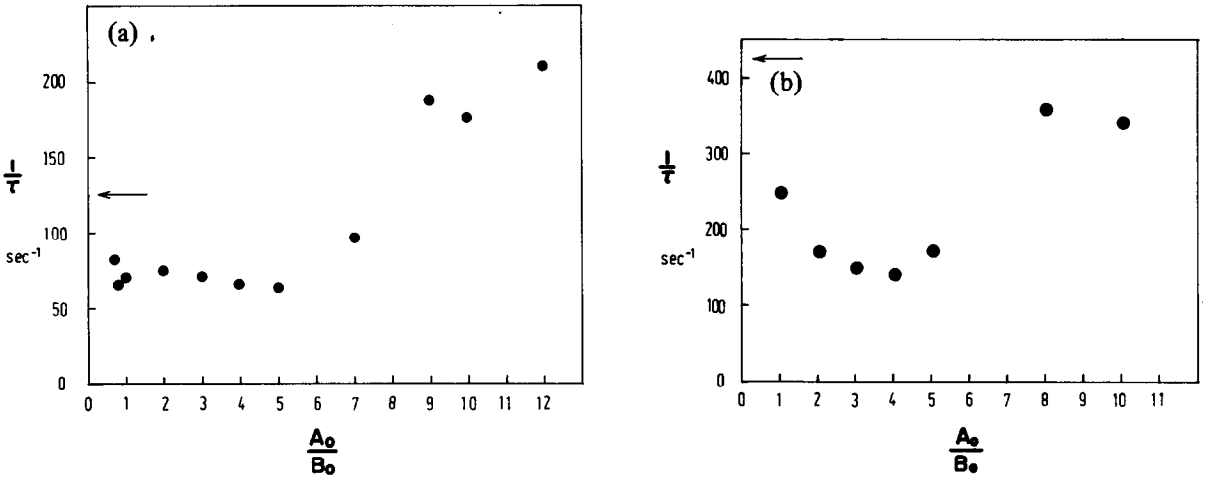

Fig. 4. Plot of $1/\tau$ vs. A_0/B_0; $A_0 = 5 \times 10^{-5}$ M (fixed). The arrow at $A_0/B_0 = 0$ shows the value of $(k_2 + k_{-2})$ expected from fig. 3. and at higher $(B_0 - A_0)$ (see text). (a) pH 7.0, (b) pH 5.2.

saturated by ANS, hence $\bar{A}=A_0-5B_0$, $\bar{B}=0$, and $n=5$. Then Eq. 5 becomes:

$$1/\tau_2 = \frac{k_2\,(A_0-5B_0)^5}{k_{-1}^5/k_1^5) + (A_0-5B_0)^5} + k_{-2}. \qquad (7)$$

When $(A_0-5B_0)^5 \gg k_{-1}^5/k_{-1}^5$, $1/\tau_2$ equals (k_2+k_{-2}). Therefore, when A_0 is fixed and A_0/B_0 is increased (i.e., B_0 is decreased), it is expected that $1/\tau_2$ decreases from (k_2+k_{-2}), down to k_{-2} (in the range $A_0/B_0 < 1$), stays constant (in the range $1 < A_0/B_0 < 5$), and then increases with A_0/B_0 to reach a plateau at (k_2+k_{-2}) (in the range $A_0/B_0 > 5$). The experimental results shown in figs. 4 and 5 are in agreement, at least qualitatively, with this prediction.

At $A_0/B_0 > 3$, the second, slower process accompanied by small increase in fluorescence was observed. The relaxation time of this process was 670 msec independent on A_0/B_0 and pH.

However, since it has not been well characterized yet, we will not attempt to include this process in the reaction mechanism represented by Eq. 5. Further kinetic studies of BSA-small molecule interactions would be useful to deduce a rational mechanism. Stopped-flow experiments using other fluorescent dyes are under study.

Acknowledgement

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