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Conductance stopped-flow study on biological complexations
Hapten-antibody and enzyme-inhibitor systems

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SUMMARY

Conductance stopped-flow (CSF) technique is used to investigate the complexations of proteins with complementary ligands. Hapten-antibody and trypsin-trypsin inhibitor systems are adopted as typical complexation reactions containing protein molecules. Clear and rapid conductance changes are observed in both systems and the reaction rate constants evaluated from the observed curves are in a good agreement with the literature values obtained by other methods, which clearly shows a usefulness of the CSF technique for the kinetic analysis of biological systems.

INTRODUCTION

Complexation of protein molecules with small molecules is an essential process in biological phenomena such as enzyme- , immunological- and energy-transfer reactions, for example. However many complexation reactions do not show spectral changes large enough to be observed. In such cases, it is very difficult to follow fast complexation reactions directly. Conductance stopped-flow (CSF) technique is very useful to observe such invisible ionic reactions.

Previously we reported association equilibria of ionic surfactant molecules (1), associations of macroions with metal ions, neutral polymer or oppositely charged macroions (2-5), complexations of metal ions with cryptands (6) and a complexation of oppositely charged latex particles (7).

Here we report the direct conductometric observation of biological association reactions for the first time, i.e., (1) the immunological reaction of antibody with low molecular weight complementary compounds, so-called "hapten", and (2) the complexation of trypsin with soy bean trypsin inhibitor (STI).

EXPERIMENTAL

Materials

a.Anti-2,4-Dinitrophenyl Group-IgG 200 mg of sodium 2,4-dinitrophenylsulfonate (50 % H_2O, Tokyo Kasei Co.) was coupled with 1 g of ovalbumin (Sigma) at pH 9.1 (adjusted by Na_2CO_3) and room temperature for 1.5 hr. and at 4°C overnight. The product, 2,4-dinitrophenyl group-containing ovalbumin (DNP-OVA, 4.1 DNP groups were bound to an OVA molecule on the average) was purified by a gel permeation chromatography using a

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Sephadex G-25 column and saline as eluting solution and lyophilized. DNP-OVA was bound to Sepharose 4B gel using BrCN activation method at pH 12 (160 mg/100 ml Sepharose gel). Anti-2,4-dinitrophenyl group-immunogloblin G (Anti-DNP IgG) from rabbit was purchased from Miles Co., and purified by the conventional procedures (precipitation by 40 % saturated ammonium sulfate, fractionation with a DEAE-cellulose column using pH 8.0, 0.01 M phosphate buffer as eluate, and immunological chromatography with the DNP ovalbumin Sepharose 4B column using 13.6 μM DNP-L-lysine (Tokyo Kasei Co.) as eluate. Immunological activity of the purified protein was checked by the Ouchterlony method using a Miles double immunodiffusion disc after each purification step.

b.Other Materials Nitrazine Yellow (2-(2,4-dinitrophenyl)-1-naphthol-3,6-disulfonic acid, disodium salt) was obtained from Sigma. Trypsin and soy bean trypsin inhibitor(STI) were purchased fron Sigma and Miles, respectively, and STI was further purified by a gel permeation chromatography (Sephadex G-75) using pH 8.50 1/3000 M tris-HCl as eluate. Elution of protein from the Sephadex column was monitored using an Atto mini-uv monitor type II (Atto Co., Tokyo. Japan). Deionized water was distilled just prior to use.

Kinetic Procedures

Conductance changes due to the complexations of proteins with complementary ligands were followed by the conductance stopped-flow apparatus. Details of the apparatus were described elsewhere (1).

The complexation of trypsin with STI was also monitored by the uv stopped-flow spectrophotometer (RA-1200, Union Engineering, Hirakata, Japan) at 260 nm (7).

RESULTS AND DISCUSSION

A. Complexation of the Anti-DNP IgG with the Hapten

By mixing a Nitrazine Yellow solution with an equal volume and concentration of Anti-DNP IgG solution, a clear relaxation curve was observed using the CSF method (Figure 1(a)), where the ordinate corresponds to the conductance change in arbitrary

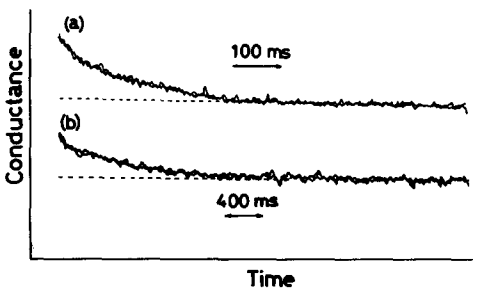

Figure 1. Typical traces of conductance changes by the complexation of proteins with complementary ligands.
(a)Anti-DNP IgG + Nitrazine Yellow at 15 °C.
[reactant]=1.15 μM.
(b)Trypsin + STI at 25 °C. [reactant]=1.0 μM.

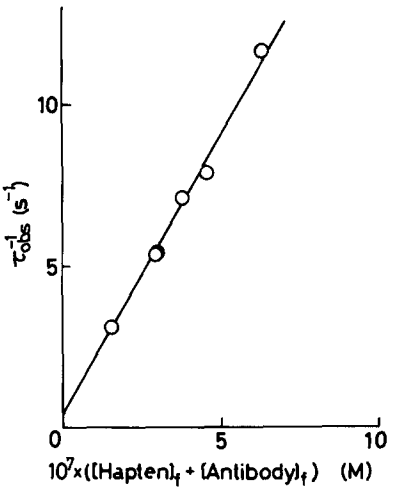

Figure 2. τ_{obs}^{-1} vs. $[Hapten]_f+[Antibody]_f$ plots at 25 °C. pH 6.0.

unit. The curve was attributed to the complexation of the antibody with the hapten, because no change in conductance was observed in the blank experiments where only the antibody or the hapten was present. From the curve we could estimate the observed relaxation time τ_{obs} for this reaction system. Varying the initial reactant concentrations (Figure 2) we could estimate the forward and backward reaction rate constants (k_f and k_b) of this reaction system from the scheme (I) and equation 1, where $[A]_f$ denotes the equilibrium concentration of the species A which was obtained by the static spectrophotometric method at 280 nm.

$$\text{Antibody} + \text{Hapten} \underset{k_b}{\overset{k_f}{\rightleftharpoons}} \text{Complex} \qquad \text{(I)}$$

$$1/\tau_{obs} = k_f\,([\text{Antibody}]_f + [\text{Hapten}]_f) + k_b \qquad (1)$$

Values obtained from Figure 2 were 1.8×10^7 $M^{-1}s^{-1}$, 0.5 s^{-1} and 3.6×10^7 M^{-1} for k_f, k_b and K (k_f/k_b), respectively. These values were in a good agreement with literature values for hapten-antibody systems(K was 5.7×10^7 M^{-1} for Anti-DNP IgG and Nitrazine Yellow (9)).

From the kinetic measurements at various temperatures, we could estimate the activation parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ to be 7.8 ±0.2 kcal.mol^{-1} and +1±1 cal.mol^{-1}.deg.$^{-1}$, respectively. The activation parameters for the reaction of anti-fluorescein IgG with fluorescein were reported to be 7.1 kcal.mol^{-1} and +0.96 cal.mol^{-1}.deg^{-1}, respectively (10). $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ of anti-DNP antibody - DNP-NH-CH_3 reaction and anti-DNP antibody - DNP-NH-n-C_4H_9 reaction were reported to be 6.4 kcal.mol^{-1}, +2.9 cal.mol^{-1}.deg^{-1} and 6.6 kcal.mol^{-1}, +2.7 cal.mol^{-1}.deg^{-1}, respectively (11). These literature values show similar tendencies to the results obtained here. The reaction observed here is one of the bimolecular association reactions and accompanied with the decrease in the entropy of activation in

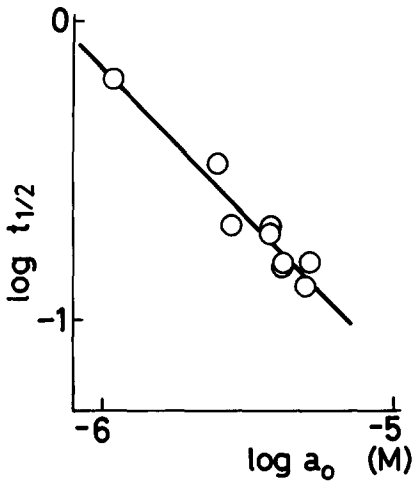

Figure 3.
Plots of log $t_{1/2}$ vs. log a_o at pH 6.5.

general. The $\Delta S^{\ddagger}$ value observed here was, however, a small positive value probably because of the compensation by the desolvation of a binding site of the antibody. Immunological association reaction is well known to be very rapid (12). $\Delta H^{\ddagger}$ value obtained is, however, larger than that of the diffusion controlled reaction (the activation energy of diffusion in water was 4.7±0.3 kcal.mol^{-1}, (13)) which also suggests that the desolvation of reactant is a rate-limiting process in the association reaction observed here.

B. Complexation of Trypsin with STI

Figure 1(b) shows a conductance change by the mixing of trypsin solution with an equal volume and concentration of STI solution. In the blank experiments where only STI or trypsin is present, no change in conductance was observed. The half-life time $t_{1/2}$ obtained was plotted against the concentration of STI in Figure 3. Luthy et al. proposed the complexation mechanism of trypsin* with STI* to be four-step (scheme (II))(8), where T, I, L, C, L* and I* denote trypsin, inhibitor, unstable complex, stable complex, modified complex and modified inhibitor, respectively.

$$T+I \rightleftharpoons L \rightleftharpoons C \rightleftharpoons L^{*} \rightleftharpoons T+I^{*} \qquad \text{(II)}$$

The equilibrium constant of STI-trypsin complex formation is so large that the complex formation can be assumed to be irreversible. Therefore the scheme (II) could be simplified as scheme (III).

$$T+I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} L \xrightarrow{k_2} C \qquad \text{(III)}$$

When the initial concentration of reactants is very low the relation between $t_{1/2}$ and the initial reactant concentration a_o is given by an equation (3) using an equation (2), where K_L is equal to $(k_{-1}+k_2)/k_1$.

$$(k_2/K_L)t = 1/a - 1/a_o \qquad (2)$$

$$t_{1/2} = K_L/k_2a_o \qquad (3)$$

The slope in Figure 3 is equal to minus one which is consistent with the equation (3). Using the equation (3) and k_2 value (50 s^{-1}, (8)) K_L could be evaluated to be 1.8×10^{-5} M which is in a good agreement with the literature value (2.3×10^{-5} M, (8)).

In conclusion, CSF technique is found to be very useful to observe fast association reactions containing protein molecules.

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