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A Stopped-Flow Kinetic Study on the Binding of Phenylbutazone to Human Serum Albumin Using Absorption and Circular Dichroism Techniques

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The mechanism of the binding of phenylbutazone(4-butyl-1,2-diphenyl-3,5-pyrazolidinedione) to human serum albumin has been investigated by kinetic experiments using both optical absorption and circular dichroism (CD) stopped-flow methods. Three distinct processes were found to exist by the absorption technique; one of these was within the dead time of the apparatus and other two slow processes were at around 10^{-2} and 1 s, of which the relaxation times were determined. On the other hand, only two processes, which correspond in time range to the first and third processes seen by optical absorption, were found using CD technique. By examining the concentration dependence of the reciprocal relaxation time of each process and by taking account of the binding isotherm, a binding mechanism has been proposed. According to the elucidated reaction scheme, successive binding of two molecules of phenylbutazone is followed by a conformational change which destabilizes one of the bound species. The detailed nature is discussed in the light of the circular dichroism change of each process.

The anti-inflammatry agent phenylbutazone has a high affinity for albumin, and is present predominantly in the bound state in serum.[1] Therefore, the binding of phenylbutazone to albumin plays an important role in the activity, metabolism and excretion of this agent in the body. In connection with these physiological significances, the binding characteristics have been investigated by many static methods such as equilibrium dialysis,[2] spectrophotometric titration,[3] fluorescence titration,[3,4] circular dichroism (CD) titration,[2,5] and spin labeling.[6] From these studies, it has been suggested that hydrophobic interactions play an important role in the binding of phenylbutazone to albumin. Furthermore, from the view point of pharmacology, competitive or cooperative binding of phenylbutazone, fatty acids and other drugs with albumin have also been studied.[7-9]

In contrast to the large amount of static information available, there has been no kinetic study on phenylbutazone binding to albumin, which is necessary in order to gain a profound understanding of these interesting phenomena.

In the present study, the binding mechanism between phenylbutazone and human serum albumin was investigated by the stopped-flow method using absorption as well as CD detection in order to obtain a detailed picture of the binding mechanism from different optical properties.

Experimental

Materials. Human serum albumin (HSA, essentially fatty acid free: less than 0.005%) was purchased from Sigma Chemical Company, and used without further purification. The molar concentration of the protein was determined by spectrophotometrically[10] Phenylbutazone(4-butyl-1,2-diphenyl-3,5-pyrazolidinedione) was also purchased from Sigma Chemical Company. A stock solution was prepared by dissolving the agent in a small quantity of 0.1 M†† NaOH solution, and then mixing with a 0.1 M phosphate buffer of pH=7.4. The concentration of phenylbutazone was determined using the molar extinction coefficient $\varepsilon_{267}=2.0\times10^4\ \mathrm{cm^{-1}\,M^{-1}}$.[3] The concentration of HSA was 3×10^{-5} M for all measurements. (In the stopped flow experiments, this concentration corresponds to the final one).

Equilibrium Dialysis. An Oxford type dialysis cell made of acryl resin was used. The volume of each compartment was 4.5 ml and it was separated by a semipermeable cellulose membrane, purchased from Visking Company. The contact area was 9.6 $\mathrm{cm^2}$. One compartment was filled with HSA solution, and the other with phenylbutazone solution. The cell was continuously rotated at 15 rpm in thermostated water at 20 °C. After 36 h, the concentration of the phenylbutazone solution was measured spectrophotometrically.

Apparatus. The absorption and CD spectra were measured with a spectrophotometer (Union Giken SM 401) and a dichrograph (Jobin-Yvon Mark III-J), respectively. The kinetic measurements were carried out using a stopped-flow apparatus (Union Giken RA 401 and RA 1100) of which dead time is 3 ms. The CD detection in the stopped experiments has been performed by combining the stopped flow apparatus with the CD spectrometer.[11] All the measurements were carried out at 20±1.2 °C.

Results and Discussion

Kinetic Data. The binding of phenylbutazone to HSA generates positive change in absorbance and CD around 285 nm;[2,5] which are, respectively, considered to be due to hydrophobic environment around phenyl

†† 1 M=1 mol $\mathrm{dm^{-3}}$.

rings of phenylbutazone at the binding site[2] and perturbation of the carbonyl chromophore of the drug by an asymmetrical locus at the site.[2,5] The present measurements were performed at 285 nm and results were similar to those in literature.[2,5]

Two distinct reaction processes were observed by optical absorption detection; typical time courses of them are shown in Fig. 1. By comparing the magnitude of the corresponding absorbance change after the dead time with that found under static conditions, another very rapid process was found to be exist within the dead time of the apparatus. In all, three phases of the kinetic processes were accompanied by a decrease in transmittance. These processes were named, in increasing order of relaxation time (τ), as the first (unobserved one within the dead time; $\tau_1<3$ ms), the second ($\tau_2\approx10$ ms) and the third ($\tau_3\approx1$ s) process. In the case of CD detection, however, only one process with a negative CD change was observed (Fig. 2). This process corresponds in time to the third one observed by absorption detection. But no relaxation corresponding to the second process was observed even by accumulating 1000 sets of data. Instead, another very rapid process with a "large positive" CD change was found to exist also within the dead time by the similar method as in the absorption detection experiments. From these observations, it can be seen that in the absorbtion detection, three kinetic processes comparably contribute to the static absorption change, but in the CD detection the unobservable first process within the dead time of apparatus dominates the static CD

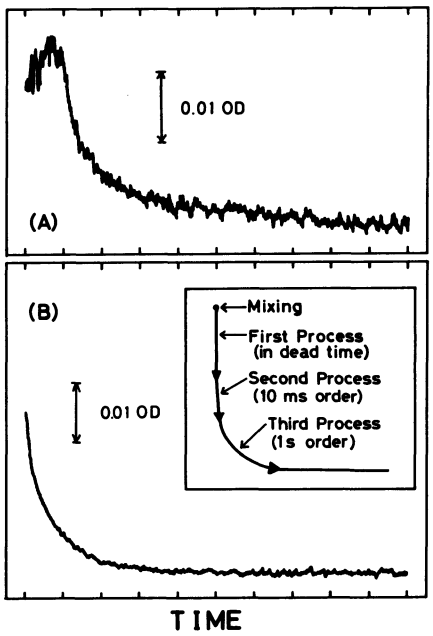


Fig. 1. Typical traces of the transmittance change at 285 nm after the mixing of phenylbutazone (1.5×10^{-5} M) and HSA (6×10^{-5} M). (A) 40 ms/div., (B) 4 s/div. The insert shows a schematic illustration of the relative scale of the transmittance change due to each process.

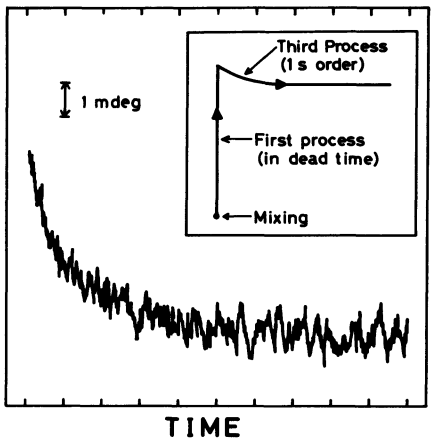


Fig. 2. A typical trace of the CD change at 285 nm after the mixing of phenylbutazone (1.5×10^{-5} M) and HSA (6×10^{-5} M). The horizontal scale is 4 s/div. The data was obtained by averaging over 40 experiments. The insert represents a schematic illustration of the relative CD change due to each process.

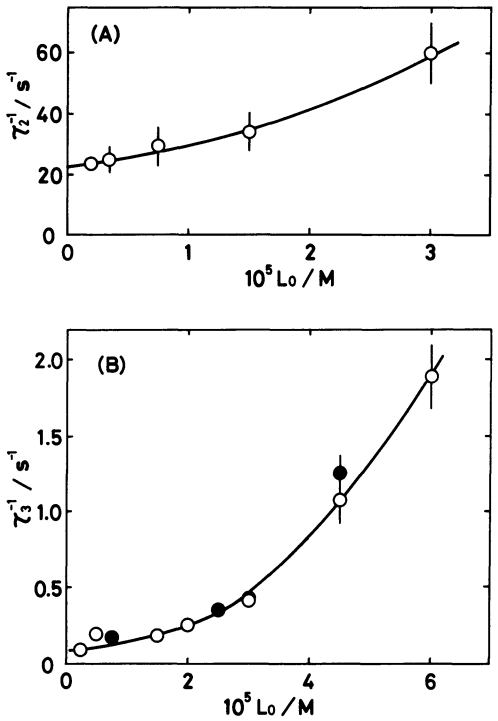


Fig. 3. The total ligand concentration (L_0) dependence of the reciprocal relaxation times of the second and third processes. (A) The second process, and (B) the third processes. (○): Determined from the change in transmittance, (●): determined from the change in CD. The solid lines are drawn to emphasize the tendencies.

change. The inserts in Figs. 1 and 2 show schematic illustrations of the relative size of the transmittance and CD changes due to these processes. The ligand concentration dependence of τ for the second and third processes is shown in Fig. 3. As can be seen in Fig. 3(B), the relaxation times of the third process obtained by the absorption and CD measurements agree well with each other.

The Binding Isotherm. A plot of $\nu/[L]$ versus ν, according to the conventional Scatchard equation; $\nu/[L]=K(n-\nu)$ where ν is the moles of ligand bound per mole of macromolecule, [L] the concentration of free ligand, K the binding constant and n the number of binding site, provides information about binding ability of the macromolecules.[12] A Scatchard plot for the present system was curved (Fig. 4), indicating that there are at least two kinds of binding site. On the conventional assumption that these binding sites are independent of each other and have an integral number of binding sites, a least squares fit analysis according to next equation; $\nu/[L]=\sum K_i(n_i-\nu_i)$ yields the binding parameters: $n_1=1$, $K_1=1.7\times10^6\ M^{-1}$, $n_2=3$, and $K_2=2.0\times10^4\ M^{-1}$, where the subscripts 1 and 2 refer to the primary and secondary binding sites. The values of the binding parameters given in the literature[2-4] differ from each other depending on the method and conditions used to obtain them. The present results are compatible with those obtained by Chignell and Starkweather[2] except for a somewhat larger value of K_1 for the present case, which is probably due to a difference in temperature and/or fatty acid content of the sample.

Analyses of the Kinetic Data. (i) The First and Second Processes: In the analyses of the stopped flow data, each kinetic process must be analyzed in increasing order of time range of the observed process because the law of mass conservation is hold at each step. Here, the second process will be analyzed taking into consideration the unobserved first process in the dead time of apparatus. For the present analysis, considering the existence of at least two kinds of binding site as suggested from the Scatchard plot, analyses were limited to models which involve more than one binding step. The following three models were examined,

$$\text{Model 1:}\quad \mathrm{L}+\mathrm{P} \underset{\text{first}}{\overset{K_1}{\rightleftharpoons}} \mathrm{C}_1 \underset{k_{-2}}{\overset{K_2\ k_2}{\rightleftharpoons}} \underset{\text{second}}{\mathrm{C}_2} \qquad (1)$$

$$\mathrm{L}+\mathrm{P}' \underset{\text{first}}{\overset{K_1'}{\rightleftharpoons}} \mathrm{C}_1'$$

where L, P, and $C_i(i=1,2)$ denote free ligand, free primary binding site and complex species, respectively, and K_i, k_i and k_{-i} are the equilibrium constant, the forward and backward rate constant of the i-th step, respectively, and superscript "′" denotes the reaction on the secondary binding site of HSA. This mechanism implies the fast competitive binding of phenylbutazone to the primary and secondary sites as the first process within the dead time of apparatus and the conformational change of the complex on the primary site as the second process. Further, this model satisfies the independency of binding sites adopted in the conventional analysis of binding isotherm.

$$\text{Model 2:}\quad \mathrm{L}+\mathrm{P} \underset{\text{first}}{\overset{K_1}{\rightleftharpoons}} \mathrm{C}_1 \underset{k_{-2}}{\overset{K_2\ k_2}{\rightleftharpoons}} \underset{\text{second}}{\mathrm{C}_2} \overset{\mathrm{L}\quad K_3}{\rightleftharpoons} \underset{\text{very fast}}{\mathrm{C}_3} \qquad (2)$$

This is a cooperative binding mechanism in which a conformational change of the first complex induces a new binding site for phenylbutazone. The third step of above scheme is not observable because of the very fast establishment of its equilibrium.

$$\text{Model 3:}\quad \mathrm{L}+\mathrm{P} \underset{\text{first}}{\overset{K_1}{\rightleftharpoons}} \mathrm{C}_1 \overset{\mathrm{L}\quad K_2\ k_2}{\rightleftharpoons} \underset{\text{second}}{\mathrm{C}_2} \qquad (3)$$

This is also a cooperative binding mechanism, but without any step of conformational change. In each of Eqs. 1—3, the second step is regarded as the observed second process. Of these models, the former two models (Model 1 and Model 2) gave the minus value for the rate constant k_{-2}, and only Model 3 was found to give acceptable results. Under the condition of rapid formation of C_1, reciprocal relaxation time of the second step in Eq. 3 can be expressed by

$$\tau_2^{-1}=k_2\frac{K_1[L]([L]+4[P]+K_1[L][P])}{1+K_1([L]+[P])}+k_{-2} \qquad (4)$$

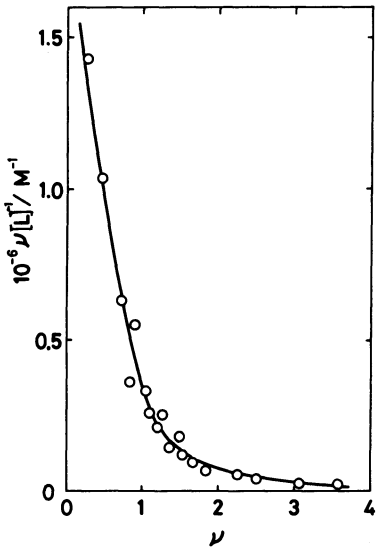
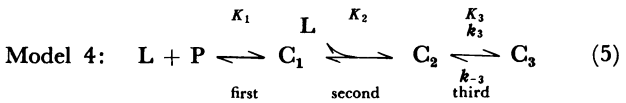


Fig. 4. The binding isotherm obtained from the equilibrium dialysis(O) and its comparison with that reconstructed from the values of the parameters associated with Model 7 (——) (see text).

where [L] and [P] are the concentration of free ligand and free binding site, respectively. This equation shows linear dependence of τ_2^{-1} on $\{K_1[L]([L]+4[P]+K_1[L][P])\}/\{1+K_1([L]+[P])\}$. [L] and [P] can be calculated by assuming values for K_1 and K_2. The plot of τ_2^{-1} versus the concentration term in Eq. 4 provides k_2 and k_{-2} as the slope and the intercept, respectively. With the guidance of linearity of the plot and self-consistent relation between parameters, the model was examined for many different values of K_1 and K_2. Figure 5 shows the plot for $K_1=(1.2\pm0.4)\times10^6\ M^{-1}$, and $K_2=(1.8\pm0.6)\times10^5\ M^{-1}$. The rate constants were determined as $k_2=(4.3\pm1.1)\times10^6\ M^{-1}$ and $k_{-2}=(23\pm6)\ s^{-1}$. The high degree of linearity suggests that Model 3 may be applied to the first and second processes. The fact that Model 1 could not satisfied the kinetic data, gives rise to question on the conventional assumption of independency of binding site which has been adopted in the binding isotherm analysis.

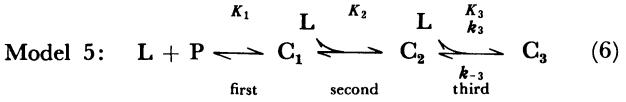
(ii) The Third Process: For the third process, the following models incorporated with the above Model 3 satisfing the first and second processes were examined.

Model 4:



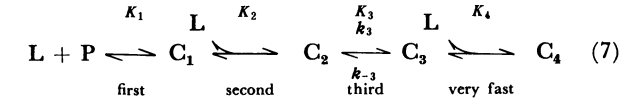
(5)

Model 5:



(6)

Model 6:



(7)

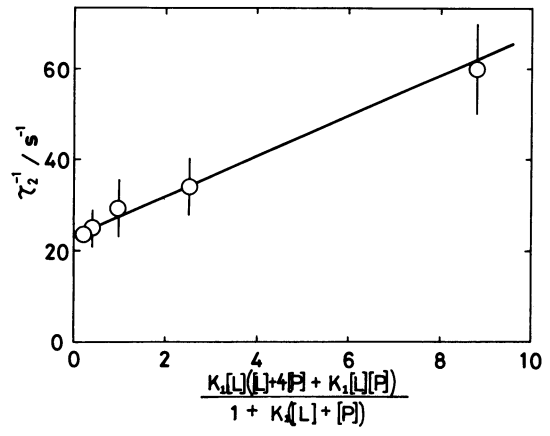
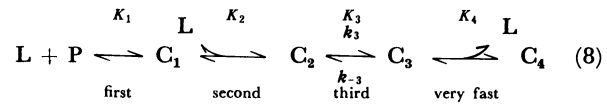


Fig. 5. Plot of τ_2^{-1} versus $K_1[L]([L]+4[P]+K_1[L][P])/(1+K_1([L]+[P]))$ for Model 3. The values on the horizontal axis were calculated using the values of the parameters cited in the text.

Model 7:



(8)

In these models, the each of third steps implies i) a conformational change of the second complex C_2 (Model 4), ii) further ligand binding to the complex C_2 (Model 5), iii) a conformational change of the complex C_2 which induces further ligand binding (Model 6), and iv) a conformational change of C_2 by which a ligand is released from the complex (Model 7). Using the values of K_1 and K_2 determined from the analysis of the second process, for each model, the concentration dependence of the reciprocal relaxation time of the third process τ_3^{-1} was examined using many sets of values of K_3 and K_4. Of these models, the first three failed from the view points of (i) the linear relationship between the observed τ_3^{-1} and the concentration dependent factor in its theoretical expression and (ii) the self-consistent relationship between the parameters. Finally, it was found that Model 7 is the only possible model for the present system. If the forth binding process in the model is very rapid, the reciprocal relaxation time for the third step in Model 7 can be expressed as

$$\tau_3^{-1} = k_3E + k_{-3}F = k_{-3}(K_3E+F) \tag{9}$$

where

$$E = (K_1K_2[L](1+K_4[L](1+K_4[L](2+K_1[L]))([L]+4[P]+K_1[L][P])+K_1K_2[L]^2(1+K_1([L]+[P])+K_1K_2K_3[L][P](2+K_1[L]))/((1+K_1[L])((1+K_4([L]+[P])+K_1K_2[L]([L]+4[P]+K_1[L][P]))+K_1K_2K_3[L][P](1+K_1[L]+K_1K_2[L]^2)),$$

$$F = (K_4[L](1+K_1([L]+[P])+K_1K_2[L]([L]+4[P]+K_1[L][P]))+K_1K_2K_3[L][P](K_1K_2[L]^2-1))/((1+K_4[L])(1+K_4[L])(1+K_1+K_1([L]+[P])+K_1K_2[L]([L]+4[P]+K_1[L][P]))+K_1K_2K_3[L][P](1+K_1[L]+K_1K_2[L]^2)).$$

Here, K_4 is defined as the association constant: $K_4=[C_3]/[L]\cdot[C_4]$. Equation 9 shows that a plot of τ_3^{-1} versus K_3E+F must be a straight line which goes through the origin, the slope of which provides the value of k_{-3}.[13] Since the linear dependency has been satisfied for some set of K_3 and K_4, all the parameters are determined so as to satisfy both the kinetic and static experimental data. The results are shown in Figs. 4 and 6. These figures demonstrate that Model 7 is full expression applicable to the present system from a kinetic and static point of view. The values of the parameters were evaluated as $K_3=(1.3\pm0.4)$, $k_3=(4.3\pm1.3)\ s^{-1}$, $k_{-3}=(14\pm4)\ s^{-1}$, and $K_4=(1.0\pm0.3)\times10^5\ M^{-1}$. These values and those associated with the

Table 1. Equilibrium and Rate Constants for Model 7

K_1 / 10^6 M^{-1}	K_2 / 10^5 M^{-1}	k_2 / 10^6 M^{-1} s^{-1}	k_{-2} / s^{-1}	K_3	k_3 / s^{-1}	k_{-3} / s^{-1}	K_4 / 10^5 M^{-1}
1.2±0.4	1.8±0.5	4.3±1.3	23±6	0.3±0.1	4.3±1.3	14±4	1.0±0.3

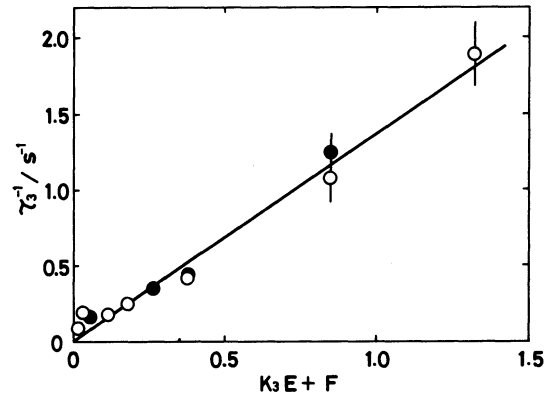


Fig. 6. Plot of τ_3^{-1} versus K_3E+F in Eq. 9 calculated using the values of the prameters cited in the text.

faster processes are summarized in Table 1. From the persent analysis of the kinetic data as well as the static one, it was clarified that the binding of phenylbutazone to HSA is cooperative binding but not the isolated binding which has been reported so far on the basis of only the static experiments.

The Binding Mechanism. From the location of the ellipticity band (287 nm), the CD change for the present system has been interpreted by asymmetrically perturbed carbonyl chromophore of phenylbutazone at the binding site.[2,5] The large positive CD change associated with the first process suggests that phenylbutazone in C_1 is already in highly asymmetrical enviroment. The binding of the secondary ligand to C_1 without any distinct conformational change suggests that the primarily bound phenylbutazone together with its environment may form a site advantageous for the secondary binding. The failure to detect any CD change associated with the second process seems to show that the secondarily bound phenylbutazone exists in a symmetrical environment. The complex C_2 thus formed, then, is converted into C_3 by rearranging of the bound ligands showing small negative CD change and thereby destabilizing one of the two bound states.

In general, and extrinsic Cotton effect reflects the state of an asymmetrically perturbed chromophore. Particularly, a close and rigid contact with an asymmetric center, which exerts an electrostatic force on the chromophore, is effective.[2,14] For the present system, the complex, C_1, which has a predominant contribution to the static CD change, may be formed by such an electrostatic interaction as well as a hydrophobic interaction.[2,3] The relatively large value of K_1 (1.2×10^6 M^{-1}) could be realized by such a dual nature of interaction.

In recent years, the extrinsic Cotton effect has frequently been used in studies of ligand binding properties of albumin.[16-22] Sometimes, a binding isotherm constructed from the CD data has been compared with that obtained from other methods.[20,21] In some cases, a relatively good agreement has been obtained through the crude assumption that all bound species give the same Cotton effect. At other times, drug interactions with albumin molecule have also been investigated using the extrinsic Cotton effect.[17,21] However, as shown by the present study as well as the kinetic studies which have been performed previously[15,23-27], the mechanism of binding between albumin and ligand is not simple but complicated involving several conformational changes. Hence, it should be noted that a deep understanding of the mechanism of complex phenomena such as drug interactions with albumin is difficult without intensive works. In this connection, kinetic studies like the present one may provide important information. Especially, the use of CD may be informative about the bound states of ligands.

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