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Kinetics and mechanism of the interaction between human serum albumin and monomeric haemin

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The interaction of human serum albumin with monomeric haemin has been investigated by detailed kinetic analysis in dimethyl sulphoxide/water (3:5, v/v). The results obtained under conditions of albumin saturation of haemin and under pseudo-single turnover conditions indicate that methaemalbumin is formed in a two-stage, single-intermediate process. The initial association between the haemin and human serum albumin is a chemically controlled process ($k_1 = 1.7 \times 10^5 mol^{-1} \cdot s^{-1} \cdot dm^3$ at 24°C); the variation of k_1 with pH exhibited a well defined pK of 5.9. The overall equilibrium constant, calculated by using microscopic rate constants, is 1.1 (± 0.5) $\times 10^8 mol^{-1}$ at 24°C. The data and conclusions are consistent with a general binding mechanism for albumin in which intermediate formation is followed by an entropy-controlled internalization of the ligand.

Albumin binds a number of hydrophobic physiologically-important ligands. Particularly detailed studies of the kinetics of interaction of bilirubin and fatty acids have been reported (Gray & Stroupe, 1978; Scheider, 1979). The binding of haemin to human serum albumin (hereafter referred to as albumin) to form methaemalbumin is of interest, not only from the aspect of protein–ligand interaction, but also from the pathological point of view, since, under certain conditions such as intravascular haemolysis or massive extra-vascular haemolysis, albumin strips haemin from methaemoglobin and transports it to the liver for further metabolism and excretion.

The kinetics and mechanism of the interaction of haemin with albumin have not, as yet, been investigated in detail. Difficulties have been encountered in studying the haemin–albumin interaction in aqueous solution, mainly due to the fact that haemin exists as aggregates in such solvent systems (Muller-Eberhard & Morgan, 1975). Any kinetic interpretation of binding data will thus be complicated by simultaneous disaggregation of haemin oligomers. Equilibrium studies on haemin–albumin interaction have been carried out by Beaven *et al.* (1974), who have demonstrated the existence of one high-affinity haemin binding site ($K_a = 5 \times 10^7 mol^{-1}$) and a number of weaker binding sites. The high affinity site was specific for haemin binding, but the weaker sites also bound fatty acid anions. The number of these non-specific binding sites was estimated to be approx. ten.

Comparative kinetic studies on the interaction of a number of metalloporphyrins (including haemin) with albumin in aqueous solution have been carried out by Parr & Pasternak (1977), and a multiphasic kinetic binding process was observed. However, in these studies, a defatted albumin preparation was used, and possible kinetic complications, due to slow interactions between the haemin and weak binding sites, cannot be excluded (see the Discussion), in addition to problems of haemin oligomer formation mentioned above.

We have studied the interaction of haemin with albumin in Me_2SO/water (3:5, v/v) mixtures, in which albumin is undenatured (by c.d. measurements) and haemin is monomeric (Beaven *et al.*, 1974; Collier *et al.*, 1979). Both relipidated albumin and native crystalline albumin have been studied. The kinetics of methaemalbumin formation indicate a two-stage single-intermediate process. The effect of both temperature and pH on the microscopic rate constants for the process have been measured. Results indicate that the first stage of the reaction may involve ligation of the haemin to a histidine residue, followed by an entropy-controlled conformational change of the intermediate to give methaemalbumin. We also present the first experimental application of asymptotic kinetic procedures to the analysis of complex protein–ligand interactions. This work was presented in part at the

Abbreviations used: Me_2SO, dimethyl sulphoxide.

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second International Symposium on the Mechanisms of Reaction in Solution, 1979 (Adams & Berman, 1979).

Materials and methods

Haemin was obtained from Sigma and from BDH and was used without further purification. Both products behaved identically. Stock solutions of haemin were prepared daily in pure, dry Me_2SO and were kept frozen at 0°C until used. Working solutions were prepared by diluting stock solutions to Me_2SO/water (3:5, v/v) and adjusting the pH to the desired value with strong (approx. 0.5 M) acid or alkali in Me_2SO/water (3:5, v/v).

Human and bovine serum albumin (fraction V) were obtained in the crystalline and defatted crystalline forms from Miles Laboratories, Goodwood, Cape Province, Republic of South Africa. Albumin preparations used in this work contained less than 5% dimer, as determined by gel filtration on Sephadex G-200. The results obtained therefore refer to the monomeric form of the protein. Relipidated albumin was prepared by dissolving defatted albumin in Me_2SO/water (3:5, v/v) and the solution was adjusted to the required pH. A 10-fold molar excess of sodium oleate was added, and fine adjustment of pH was made if necessary. The defatted protein was readily soluble in the Me_2SO/water mixtures used.

Human serum albumin (not defatted) dissolved very slowly in Me_2SO/water (3:5, v/v), and was therefore dissolved first in distilled water, before slow addition of Me_2SO to the required solvent composition. The pH of the solution was then adjusted as before. During addition of Me_2SO the temperature was kept below 25°C by cooling as required.

Solutions used in this study were unbuffered because of the insolubility of salts in the Me_2SO/water mixtures used. The apparent pH of all solutions used was adjusted before, and checked after, the reaction, by using a glass electrode. pH values were stable: the maximum difference between the two values was 0.05 pH unit.

Highest purity Me_2SO (Merck and BDH grades) was used in all the studies reported here. The pure Me_2SO, which was colourless and virtually odourless, was stored at 0°C. The two preparations gave identical results.

Reactions were monitored by using an Aminco DW-2 dual wavelength spectrophotometer fitted with a Morrow–Chance stopped-flow attachment. Absorbance–time data were digitized and stored by using a DASAR data acquisition system (Aminco).

Single and multi-exponential functions were fitted to experimental data by non-linear iterative regression techniques. For multi-exponential functions, initial parameter estimates were obtained with a grid-search procedure, and these were then refined by using standard non-linear regression techniques.

Simulation studies of the experimental system were carried out by a numerical integration of the sets of non-linear differential equations characterizing the mechanisms investigated. In all cases studied the sets of equations were well conditioned and integration was carried out by using a fourth-order Runge–Kutta integration procedure with variable step length (see, e.g., Tenenbaum & Pollard, 1964). The procedure proved to be rapid and accurate. The cumulative integration error was checked by using the calculable equilibrium properties of the system (see the Results section), and was found to be less than 10^{-4}% in all integrations carried out. All computations were carried out by using a Techtronix 4051 graphics system or a Data General Micro-Nova system, with programs written in BASIC.

Results

Spectral changes observed on mixing haemin with excess albumin in Me_2SO/water (3:5, v/v) at pH 7.00 are shown in Fig. 1. The $\lambda_{max.}$ values and approximate absorbance coefficients are shown on the Figure. The absorbance coefficient obtained by ourselves for haemin in Me_2SO/water (3:5, v/v) is considerably higher than the value obtained by Beaven *et al.* (1974), but is in good agreement with the values of $1.8 \times 10^5 M^{-1} \cdot cm^{-1}$ obtained by Brown & Lantzke (1969), $1.45 \times 10^5 M^{-1} \cdot cm^{-1}$ obtained in Me_2SO/ethanol mixtures by Stern & Peisach (1974), and $1.7 \times 10^5 M^{-1} \cdot cm^{-1}$ obtained by Collier *et al.* (1979) for monomeric haemin in Me_2SO/water (7:13, v/v). These last authors have suggested that the low absorbance coefficient observed by Beaven *et al.* (1974) could be due to impurities in the Me_2SO used. However, since only commercial high-purity Me_2SO was used in the present study, as was apparently the case with Beaven *et al.* (1974), the difference observed does not seem to be caused by impurities in the solvent.

In the present study we have used the increase in absorbance at 423 nm to monitor the formation of complexed haemin–albumin, and the decrease in absorbance at 406 nm to monitor the decrease in free haemin concentration. The reference wavelength used was 495 nm, at which no change in absorbance was observed at any time during the reaction.

A typical set of experimental absorbance–time data are shown in Fig. 2, where the inadequacy of a single exponential function for the modelling of the binding kinetics is demonstrated by the semi-logarithmic plot of the data. In all experiments carried out under conditions of saturating albumin ($[albumin]/[haemin] > 7$), the data were adequately fitted by a biexponential function (Fig. 2). No

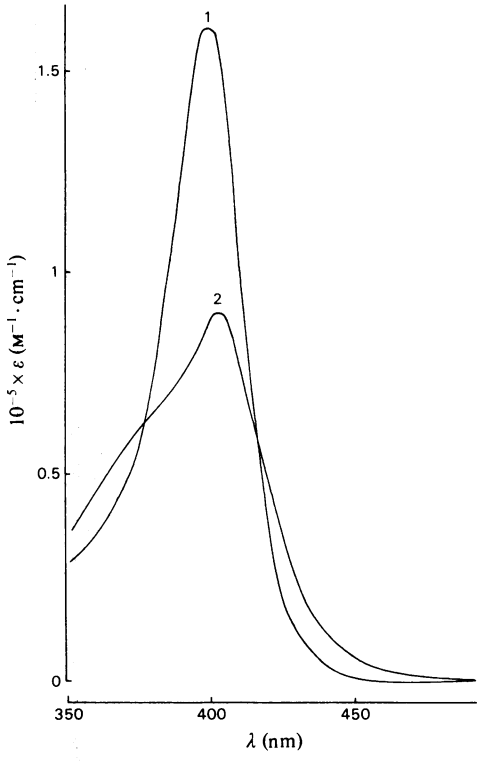

Fig. 1. *Spectral changes observed on mixing human serum albumin* (2×10^{-5} *mol·dm*$^{-3}$) *and haemin* (5×10^{-6} *mol·dm*$^{-3}$) *in* Me_2SO*/water (3:5, v/v), pH 7.00, 25°C*

Curve 1, haemin alone ($\lambda_{max.}$ 402nm); curve 2, haemin + albumin ($\lambda_{max.}$ 408nm).

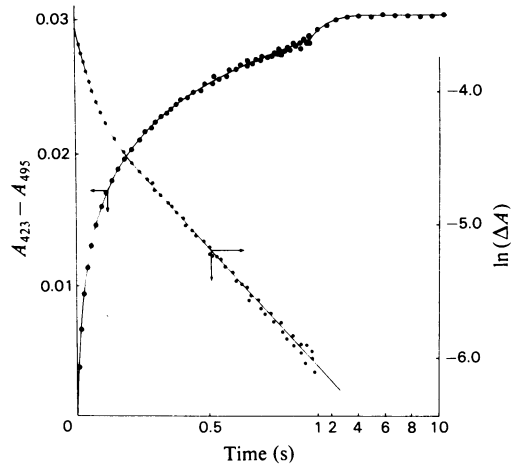

Fig. 2. *Typical reaction trace obtained on monitoring the formation of complexed haemin and human serum albumin*

[Haemin], 4.80×10^{-6} mol·dm^{-3}; [albumin], 8.50×10^{-5} mol·dm^{-3}. (●), A semilogarithmic plot of ln $(A_\infty - A)$ [ln (ΔA)] versus t is shown. The experimental points (●) are fitted by the function $A = 0.02987 - 0.01217e^{-21.8t} - 0.01732e^{-2.6t}$.

statistical improvement in the fitting of data was obtained on proceeding to a tri-exponential function. In particular we used the following, in addition to minimization of the sum square residuals, as criteria of data fitting: (*a*) the experimental points scattered randomly about the best-fit line with a 'noise' level approximately equal to the machine noise level (assessed immediately after the completion of the reaction); (*b*) the infinity value of the absorbance was closely predicted from the fitting of a biexponential function to data from the first 80% of the reaction. Fitting of a biexponential function leads to the direct evaluation of macroscopic rate constants $k_{1,obs.}$ and $k_{2,obs.}$, for the fast and slow stages of the reaction respectively. Favourable resolution of the macroscopic rate constants was possible for the biexponential fitting procedure, since the ratio of the fast to the slow constants was of the order of 10.

Fig. 3 shows plots of $k_{1,obs.}$ and $k_{2,obs.}$ versus [albumin] at pH 7.00 and 23.7°C. It is readily apparent that the concentration-dependence of $k_{1,obs.}$ is linear, whereas $k_{2,obs.}$ varies hyperbolically with [albumin] (inset to Fig. 3). These observations suggest a single-intermediate reaction (1) as the mechanism for the methaemalbumin formation process:

$$\text{Haemin} + \text{albumin} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{I} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{methaemalbumin} \quad (1)$$

where I represents the intermediate species. For the model (1), the concentration dependence of $k_{1/2\,obs.}$ is given by eqns. (2) and (3), when one reactant, namely albumin, is in large excess, and the system is treated in terms of transient-state kinetics (Halford, 1971).

$$k_{1,obs.} = k_1[\text{albumin}] + k_{-1} \quad (2)$$

$$k_{2,obs.} = \{k_2K_1[\text{albumin}]/(1 + K_1[\text{albumin}])\} + k_{-2} \quad (3)$$

where $K_1 = k_1/k_{-1}$

Eqns. (2) and (3) were fitted to the data (for which [albumin]/[haemin] > 7) in Fig. 3 by linear and non-linear regression, respectively, and gave the microscopic rate constants: k_1, $1.7(\pm 0.3) \times 10^5$ mol^{-1}·s^{-1}·dm^3; k_{-1}, $10.1(\pm 3)$s^{-1}; k_2, $6.3(\pm 0.3)$s^{-1}; k_{-2}, $0.0(\pm 0.1)$s^{-1}.

The value of k_{-1}/k_1, evaluated directly on fitting eqn. (3) to the data shown in Fig. 3(*b*), was

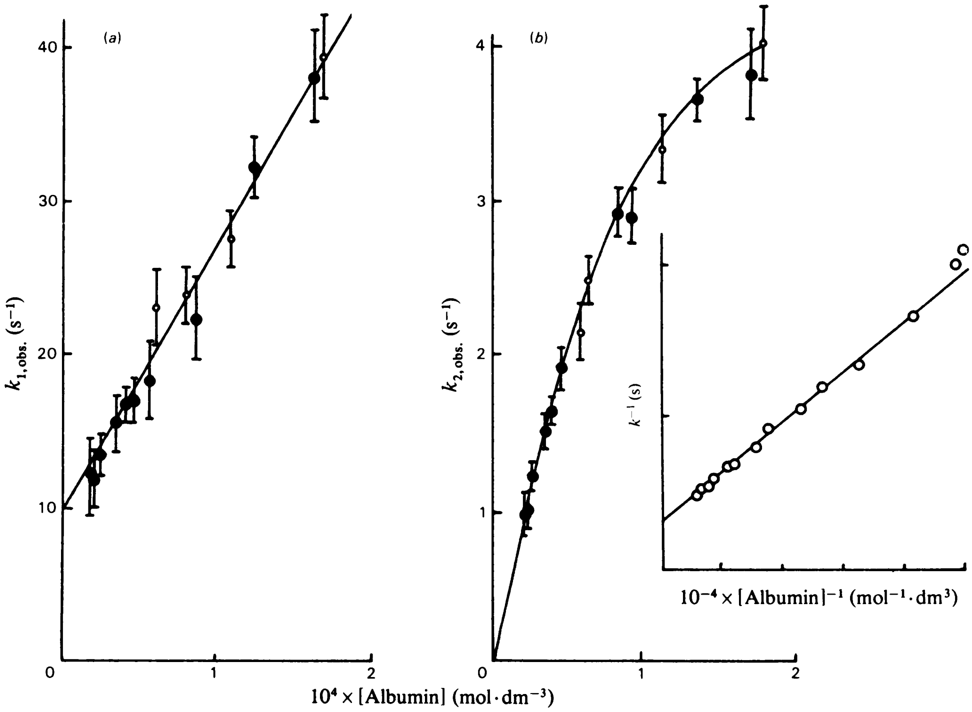

Fig. 3. *Concentration dependences of the macroscopic rate constants (obtained from the biexponential curve fits) for the interaction of haemin with human serum albumin at pH 7.00 and 23.7°C in Me_2SO/water (3:5, v/v)*

●, Relipidated albumin; O, human serum albumin (Not defatted). Error bars are 90% confidence limits for a minimum of eight repeat reactions at each concentration. The hyperbolic nature of the $k_{2,obs.}$ versus [albumin] plot is demonstrated by the inset.

$(9.2\pm0.9)\times10^{-5}$ mol·dm^{-3}. This is in good agreement with the value of $(6.0\pm3.0)\times10^{-5}$ mol·dm^{-3} calculated from the values of k_1 and k_{-1} obtained on applying eqn. (2) to the data in Fig. 3(*a*). The internal consistency of the model, which is essential for verification of mechanism (1) (Bernasconi, 1976), is thus excellent.

The fact that the concentration variation of $k_{1,obs.}$ is linear whereas that of $k_{2,obs.}$ is hyperbolic clearly indicates that the first and second stages of the reaction are being observed; no very fast chemical interaction, complete within the mixing/dead time of the stopped-flow apparatus (1–2 ms) having taken place.

Evaluation of $k_{-2,\,max.}$

A maximum value for k_{-2} can be obtained from experiments in which human methaemalbumin is mixed with a large excess of bovine serum albumin. Since bovine serum albumin reacts far less readily than human serum albumin with haemin, large molar excesses of bovine serum albumin are required to produce measurable spectral changes. The redistribution of haemin between the two serum albumins can be monitored at 403 or 423 nm, with a reference of 565 nm. The overall reaction can be represented by scheme (4):

bovine serum albumin

$$+\text{ human methaemalbumin} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} \text{haemin}$$

$$+\text{ human serum albumin} + \text{bovine serum albumin}$$

$$k_b \swarrow\!\!\nearrow k_{-b}$$

$$\text{human serum albumin} \pm \text{bovine methaemalbumin} \quad (4)$$

If the association rate-constants of haemin with human and bovine serum albumins are greater than the dissociation rate-constants, the observed process should follow pseudo-first order kinetics with a macroscopic rate constant equal to the sum of the microscopic dissociation rate constants ($k_a + k_{-b}$). A maximum value can therefore be estimated for the methaemalbumin dissociation rate-constant, k_{-2}. Fig. 4 shows a typical reaction trace for the reaction of bovine serum albumin with human methaemalbumin. The process follows pseudo-first order

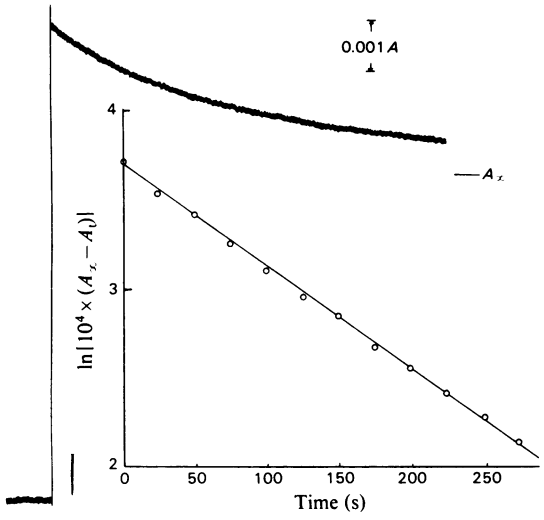

Fig. 4. *Typical relaxation curve for the redistribution of haemin between human and bovine serum albumin in Me_2SO/water (3:5, v/v) at pH 7.00*

Initial concentration of human methaemalbumin, 5×10^{-6} mol·dm^{-3} and of bovine serum albumin, 1×10^{-4} mol·dm^{-3}.

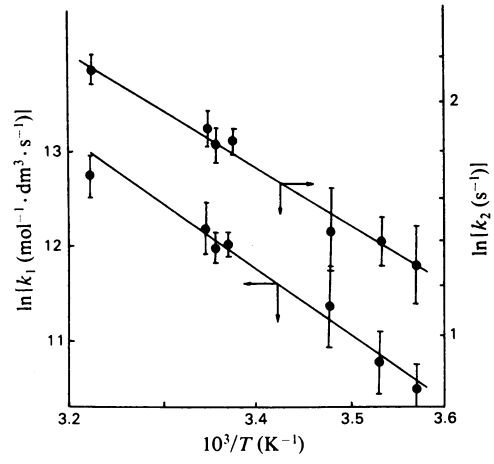

Fig. 5. *Arrhenius plots of rate constant data for the binding of monomeric haemin to human serum albumin (k_1) and for the conversion of the intermediate complex to methaemalbumin (k_2)*

All reactions were carried out at pH 7.00. The error bars indicate ± 1 S.D.

kinetics, reflecting a relaxation to a new equilibrium state. The values of $k_{-2,\,max.}$, obtained from a series of experiments at [bovine serum albumin]/[human methaemalbumin] ratios of between 7 and 20, was found to be 0.0063 (± 0.0011) s^{-1}.

Effect of temperature and pH

The effect of temperature and pH on k_1 and k_2 (evaluated as in Fig. 3) were investigated to give further insight into the mechanisms of the individual stages of the combination process. The results obtained are summarized in Figs. 5 and 6 and in Table 1.

Exact determination of k_{-2}

The maximum value obtained for k_{-2} allows calculation of only a limiting minimum value for the overall equilibrium constant ($K_{eq.}$) of the reaction. To obtain a true estimate for $K_{eq.}$, we have determined k_{-2} from a series of experiments at [haemin] = [albumin]. Under these conditions, with accurate determination of [haemin] as the reaction approaches equilibrium, two independent estimates can be obtained for k_{-2}, by using either the equilibrium concentration of haemin, or the asymptotic kinetics of the approach to equilibrium.

Equilibrium concentration approach. It can be shown for a system of type (1) that when $k_{-2} \neq 0$ and $[\text{haemin}]_0 = [\text{albumin}]_0$, then the equilibrium concentrations are given exactly by eqn. (5):

$$[\text{Haemin}]_{eq.} = [\text{albumin}]_{eq.} = \frac{-k_{-2} + \{k_{-2}^2 + 4K_0 k_{-2}[\text{haemin}]_0\}^{\frac{1}{2}}}{2K_0} \quad (5)$$

where $K_0 = (k_{-2}k_1 + k_2k_1)/k_{-1}$ and $[\text{haemin}]_0$ = the initial concentration of haemin.

We have determined the values of $[\text{haemin}]_{eq.}$ from a number of repeated experiments at an initial concentration of haemin of 6.1×10^{-6} mol·dm^{-3}, an example being shown in Fig. 7. Complete (i.e. 100%) reaction absorbance values were obtained by carrying out the reaction at identical haemin concentration, but at albumin concentrations of 0.6 and 1.2×10^{-4} mol·dm^{-3} immediately before and after the equal-concentration experiments. With these complete-reaction values the mean equilibrium concentration of haemin (obtained from four separate experiments) at $[\text{haemin}]_0 = [\text{albumin}]_0 = 6.1 \times 10^{-6}$ mol·dm^{-3} was estimated to be $1.9(\pm 0.7) \times 10^{-7}$ mol·dm^{-3} (mean ± total deviation), i.e. 96.9 ± 1.2% complete. Therefore, assuming the values of k_1, k_{-1} and k_2 given previously, k_{-2} is calculated [by using eqn. (5)] to lie in the range 0.00038–0.00109 s^{-1}.

Asymptotic kinetic approach. The treatment of single-turnover kinetics [system (1) with $k_{-2} = 0$]

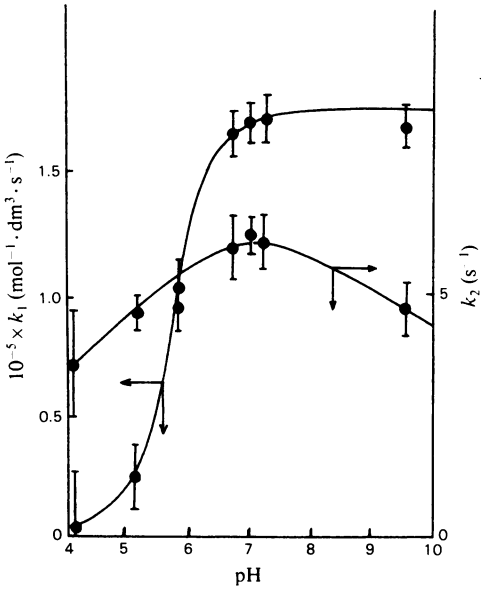

Fig. 6. *Variation with pH of the microscopic rate constants for the haem binding stage (k_1) and the intermediate rearrangement stage (k_2) in the formation of methaemalbumin*
Reactions were carried out at 25°C. The error bars indicate ± 1 S.D.

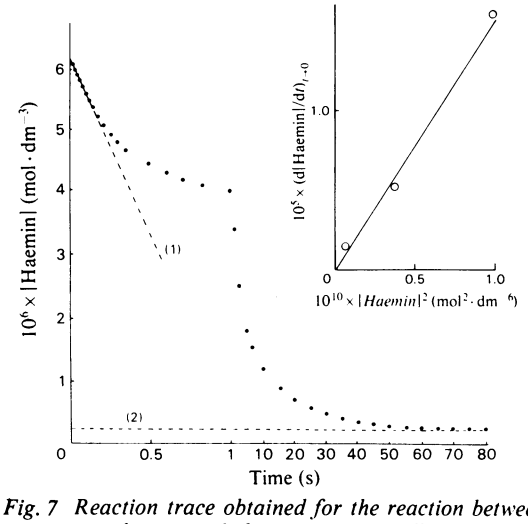

Fig. 7 Reaction trace obtained for the reaction between monomeric haem and human serum albumin under equal-concentration conditions
[Haemin] = [albumin] = 6.1×10^{-6} mol·dm^{-3}. Experimental points shown are the means for eight consecutive runs. (1) The initial slope; (2) the equilibrium concentration reached at $t = \infty$. The inset indicates the linear variation of the initial rate of haem decrease with the square of the concentrations for three equal-concentration experiments.

Table 1. *Pseudothermodynamic activation parameters for the binding (stage 1) and conformational rearrangement (stage 2) stages in the formation of methaemalbumin*

Stage	Rate constant	E_a (kJ·mol^{-1})	$\Delta S^{\ddagger}$ (J·K^{-1}·mol^{-1})
1	k_1	52.4 ± 4.3	23.1 ± 15
2	k_2	21.9 ± 2	−164 ± 7

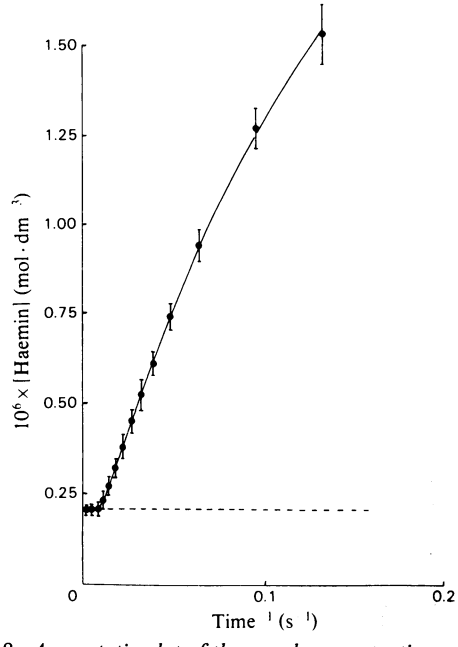

Fig. 8. *Asymptotic plot of the equal-concentration experiment shown in Fig. 7*
The intercept with the equilibrium haem concentration occurs at $t^{-1} = 0.0091 \pm 0.00090\,s^{-1}$. The broken line is equivalent to an equilibration concentration of haemin of $2.05(\pm 0.15) \times 10^{-7}$ mol·dm^{-3}. The error bars represent the error from eight repeat experiments.

has been previously considered in terms of asymptotic expansions by Adams (1977). For such systems, under equal-concentration conditions, the reactant concentrations vary with time as $t \to \infty$ as shown in eqn. (6):

$$\text{Reactant} = \frac{K_m}{k_2} \cdot \frac{1}{t} + \ldots. \qquad (6)$$

$$K_m = (k_{-1} + k_2)/k_1$$

Fig. 8 shows the asymptotic plot of the experimental data in Fig. 7. The plot does not pass through the origin, since the value of k_{-2}, although small, is finite and eqn. (6) is thus only approximately true.

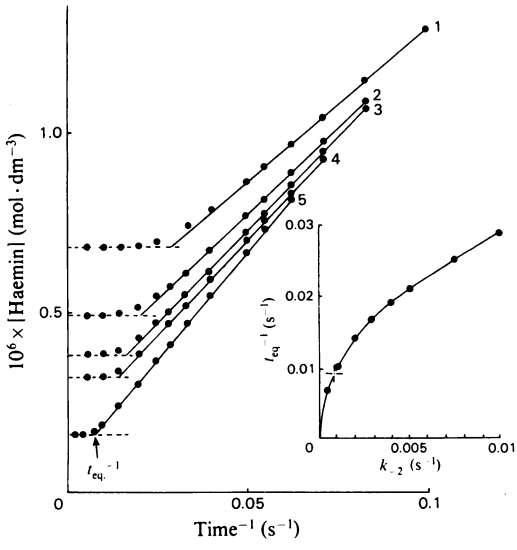

Fig. 9. *Results of simulation studies on the asymptotic behaviour of 'almost' single-turnover analogues of the methaemalbumin reactions*

Curves 1–5 have k_1, k_{-1} and k_2 as in the text, but with $k_{-2} = 0.01$, 0.005, 0.003, 0.002, and 0.0005 s^{-1} respectively. The broken lines are the calculated equilibrium [haemin] for initial [haemin] = [albumin] = 6.1×10^{-6} mol·dm^{-3}. The inset shows the variation of the intercept $t^{-1}_{eq.}$ with k_{-2}, the cross being the experimental $t^{-1}_{eq.}$ giving $k_{-2} = 0.00091 \pm 0.00011$ s^{-1}.

The intercept of the asymptotic plot with the line [haemin] = equilibrium concentration can be calculated and is 0.0091 ($\pm$0.00090) s^{-1} (mean $\pm$ 1 S.D.), from four separate experiments of the type shown in Fig. 8. This value is not affected by the error attached to the estimate of [haemin]$_{eq.}$, since the internal infinity absorbance value for equal-concentration reactions, which is used in the accurate calculation of the intercept, is accurately and precisely determinable for each experiment.

Fig. 9 shows some representative asymptotic plots constructed from computer simulation of system (1) by using the known, constant values of k_1, k_{-1} and k_2, but varying the values of k_{-2} (as shown in the Figure) in the range 0.0005–0.01 s^{-1}. The inset of the Figure shows a plot of the intercept ($t_{eq.}^{-1}$) versus k_{-2}. From this plot and the experimental value of $t_{eq.}^{-1} = 0.0091$ s^{-1}, we estimate k_{-2}, by interpolation, to be 0.00091 ± 0.00011 s^{-1}, in excellent agreement with the equilibrium value; with this value of k_{-2}, the overall equilibrium constant ($K_{eq.}$) for the process (1) is calculated to be $(1.1 \pm 0.53) \times 10^8$ mol^{-1}.

Discussion

We have demonstrated in this study that the reaction of haemin with lipidated albumin in Me_2SO/water (3:5, v/v) is a one-intermediate process with no other intermediates, either shorter or longer lived, being kinetically detectable. It is worth stating here our reasons for these conclusions: (*a*) the experimental product–time curves are adequately fitted by biexponential functions; (*b*) the concentration variation of macroscopic rate constants is consistent with that expected from the first and second stages of a process such as (1); (*c*) the total absorbance change of the process is the sum of the absorbance changes for the two kinetic processes; (*d*) the infinity and zero-time absorbances are accurately predicted by a two-stage (one-intermediate) model; (*e*) the initial rate of haemin concentration decrease varies linearly with the square of the haemin concentration for equal-concentration experiments; (*f*) the asymptotic behaviour of the kinetics is consistent with that expected for a one-intermediate process.

The overall equilibrium constant for the binding process, as calculated from the microscopic rate constants for the individual stages, is $1.1(\pm 0.53) \times 10^8$ mol^{-1}, in good agreement with the value of 5×10^7 mol^{-1} obtained from the aqueous equilibrium studies of Beaven *et al.* (1974). It is therefore apparent that extrapolation of our conclusions to the aqueous situation is validated.

The temperature variation of k_1 and k_2, combined with the effect of pH on these constants, suggest that the first stage, i.e. intermediate formation, involves the bimolecular interaction of monomeric haemin with a group on the protein surface with pK of 5.9 ($\pm$0.1). From the large negative value of $\Delta S^{\ddagger}$ for the second stage of the process, it would appear that the conversion of intermediate I to methaemalbumin involves a conformational folding of the intermediate to a more ordered form.

These conjectures are in broad agreement with the conclusions of previous workers, who envisage the interaction of haem with albumin as involving axial ligation of a specific amino acid to the porphyrin metal atom, in conjunction with a conformational rearrangement of the intermediate. We differ, however, in that our results suggest that the first stage of the reaction involves amino acid–iron interaction (the pK suggesting that the amino acid involved could be histidine, p$K_{His.}$ 6.04 in aqueous solution) on the protein surface. This intermediate then undergoes a conformational folding to a thermodynamically more stable complex. Parr & Pasternak (1977) consider however that the conformational folding in the second stage of the process brings the haemin, which is weakly bound at the surface, to the interior of the protein where it interacts with a specific amino acid residue.

The process that we have deduced bears a close resemblance to the recently-discussed mechanism of fatty acid binding to albumin (Scheider, 1979). In this case, the fatty acid residues first interact in a diffusion-controlled manner with a low-affinity site on the surface of albumin. The complex then rearranges in an entropy-controlled process and allows the fatty acid to gain access to a specific binding site in the interior of the protein.

Two points should perhaps be made here regarding the detailed nature of the initial reaction of haemin with albumin. Although the first stage of any bimolecular process must be a diffusion-controlled movement of the species together in the bulk medium, i.e. a physically controlled process, it is evident that the second-order combination of haemin with albumin is a chemically controlled process, since the rate constant is a factor of 10^3 lower than that expected for diffusion-controlled protein–ligand interaction processes.

Parr & Pasternak (1977) have detected a rapid fluorescence change in the reaction of metalloporphyrins with defatted albumin. This could either reflect the masking of a fluorescent amino acid residue by the haem as it diffuses toward the specific (?) histidine residue for reaction, the fluorescent residue being close to the reactive histidine. Alternatively, it could reflect the first rapid stage of the reaction between the metalloporphyrin and the dual-nature fatty acid/haemin binding sites, which were not oleate-titrated in their study.

As can be seen from Fig. 3 the behaviour of the native and relipidated albumins is kinetically indistinguishable. In our preliminary studies of the systems, however, we investigated the kinetics of the interaction of haemin with the delipidated albumin.

The initial rapid biphasic absorbance change was in this case followed by a slow ($t_{\frac{1}{2}}$ of the order 10^2–10^3 s) change in absorbance. This possibly reflects the slow phase of the interaction of haemin with the multiple weak fatty acid-binding sites. In contrast, all reactions of the relipidated and native albumins were kinetically clean, and showed no very slow component in addition to the two phases already discussed in the Results section. Although haemin will interact weakly with the dual nature haemin/fatty acid sites, the primary haemin-binding site, with which we have been exclusively concerned here, does not appear to interact with fatty acid residues (Beaven *et al.*, 1974). This would seem to argue against involvement of the haemin propionate residues in the binding of haemin to the primary site.

Bilirubin binding to albumin has been shown to be a three-stage two-intermediate process (Gray & Stroupe, 1978). It appears therefore that several ligands, haemin, fatty acids and bilirubin, which are known to be transported by albumin, bind to the protein in discrete stages; an initial superficial binding reaction is followed by a slower 'internalization', leading to a more stable complex. It can be speculated, therefore, that this may be a general process for the binding of ligands transported by serum albumin. The internalization provides steric protection of the bound ligand against diffusion off the carrier protein surface, thus allowing for efficient release of the bound, internalized ligand only at the target or delivery site, where the conformational internalization step can be specifically reversed.

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