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Kinetics of quinine–deuterohemin binding

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Abstract

The interaction of quinine with free hemin is of importance for the antimalarial effect of the drug in infected erythrocytes. We have investigated the kinetics of the complex formation of quinine with deuterohemin using the temperature jump relaxation method. We use ethyleneglycol–water mixtures as a solvent, where sufficient solubility for both species is provided and dimerization of the hemins, which involves μ-oxo bridges, can be controlled. Equilibrium and kinetic data for the dimerization of deuterohemin are given at 30 and 50 vol% ethyleneglycol. Binding of quinine is significantly slower than dimerization. Both processes are well separated on the time axis and yield a relaxation progress curve which is described with high accuracy by two exponential terms. The slow relaxation process is analyzed with respect to a 1 : 1 complex formation. This is the simplest mechanism which accounts for the present data, leading at 30 vol% ethyleneglycol, pH 7.5 to a binding constant of 10^4 M^{-1} and rate constants of 4.4×10^5 M^{-1} s^{-1} for the association and 44 s^{-1} for the dissociation step. However, there is evidence from the fast relaxation process that monomeric and dimeric hemin exhibit different reactivity. There is a strong decrease in rate with increasing pH. The implication of the results with respect to the proposed mechanisms of complex formation with quinoline drugs is discussed.

Keywords: Quinine–hemin binding; Hemin dimerization; Relaxation kinetics; Antimalarial drug

1. Introduction

The complex formation of the antimalarials quinine and chloroquine with hemin is suggested to be a crucial interaction for the drugs' physiological effect in allowing hemin to act as a receptor for the antimalarials in plasmodium infected erythrocytes [1,2]. Free hemin results from the degradation of hemoglobin by the parasite. It is toxic to the parasite, but is detoxified by conversion into the malaria pigment hemozoin [3]. It is suggested that hemin–drug complexes are not incorporated into the pigment [4] and very recently it has been detected that a heme polymerase enzyme is involved [5].

The formation of complexes with different hemins *in vitro* has been demonstrated with various techniques such as equilibrium dialysis [2], spectrophotometric titration [6–8] and NMR [7,9–11], but quantitative information is rather scarce, being restricted to equilibrium studies with urohemin [10,11]. We do not know of any kinetic investigations on the interaction of quinoline compounds with hemins.

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The stoichiometry of the complexes is in question. While in the early investigations [2] with chloroquine a stoichiometry of two protohemins to one chloroquine is deduced from equilibrum dialysis, later experiments in aqueous solution [8] were consistent with a 1:1 complex. A particularly complex behavior is reported for the interaction of urohemin with quinine. Constantinides and Satterlee [10] deduce from spectrophotometric data a stoichiometry of two hemins to one quinine, but at the same time find a cooperativity with respect to quinine binding, with a Hill coefficient of 2. This would require rather intricate complexes containing at least four hemins and two quinines.

In view of the importance of the hemin–quinine interaction we started to perform an investigation on the kinetics of complex formation of quinine and deuterohemin using the temperature jump relaxation method.

Investigations of hemin species in aqueous solutions are strongly influenced by the tendency of the hemins to dimerize, forming μ-oxo bridges [12,23–26]. Moreover, in neutral and weakly acid solutions of protohemin, which is the natural prosthetic group of hemoglobin, a stable state cannot be achieved, probably due to colloidal precipitation leading to artifacts in quantitative work. Dimerization is less pronounced with deuterohemin which lacks the vinyl groups [13].

We demonstrate in the present paper that the complex formation can well be followed under conditions where dimerization of the hemin species plays a significant role. This is possible since dimerization of the hemin and quinine binding are well separated on the time scale, the binding of quinine being significantly slower.

Dimerization of the hemins can effectively be modified by the addition of alcohols [14–17]. We report here on the thermodynamics and the kinetics of quinine–deuterohemin interactions in solutions containing ethyleneglycol. Under these conditions the equilibrium and kinetics of dimerization can rather well be characterized and controlled. Varying the glycol content is a means of studying the binding behavior at different monomer to dimer ratios. Our experiments are along this line.

We present a general description of the kinetic observations and give a first analysis of the relaxation times and relaxation amplitudes. Quinine binding to deuterohemin under conditions, where both monomers and dimers are present (30 vol% ethyleneglycol) can be understood with a simple 1:1 binding to hemin monomers. It is not yet definitely established to which extent hemin incorporated into dimers exhibits different reactivity.

The reaction can be followed in a wide pH range since sufficient solubility of both species in ethyleneglycol–water mixtures is given. This is another important aspect. Insolubility of quinine at alkaline pH and of the hemins at acid pH impedes investigations in aqueous solutions.

2. Materials and methods

Deuterohemin has been prepared according to the procedure given in [18] by melting together protohemin (Aldrich) and *m*-dihydroxybenzene (resorcin) at about 160°C. The formed vinyl ether is extracted with diethylether from the powdered material until the extracts are almost colourless. The remaining product is dissolved in a chloroform–pyridine mixture (3:1) and the filtered solution is added to hot glacial acetic acid containing a small amount of concentrated HCl. Fine deuterohemin crystals are formed on cooling. Stock solutions are prepared in an ethyleneglycol(95%)–water mixture containing 0.04 *M* NaOH. Quininesulfate was a product of Böhringer. The static and kinetic measurements were performed in solutions containing 0.01 *M* buffer and 0.1 *M* NaCl. Phospate, bis-tris and borate buffers have been used. The pH values were determined with a glass electrode, which was soaked and kept in aqueous (3 *M* KCl) solution and was calibrated with aqueous standard buffers. All pH-values given in this paper do therefore refer to this conventional choice of the standard state.

The spectrophotometric titrations were done by mixing different ratios of hemin solutions containing no quinine and an excess of quinine respectively. The spectra were recorded with a

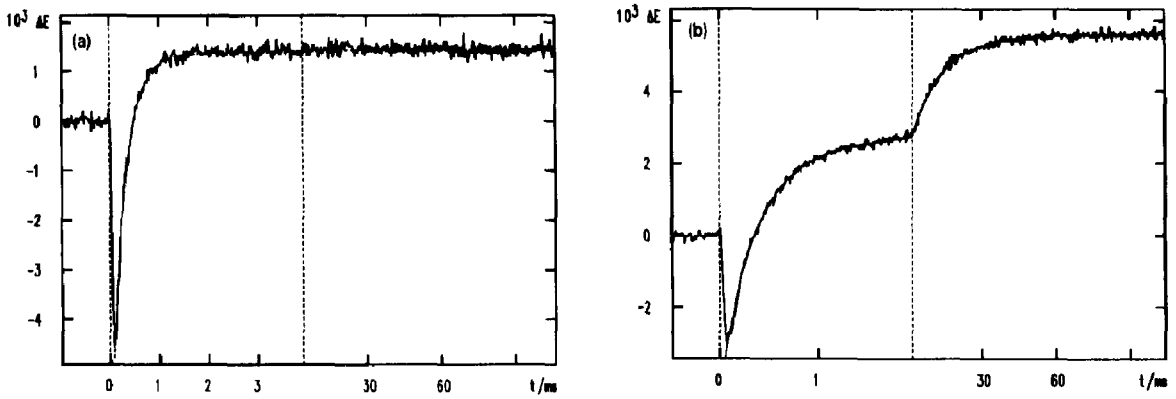

Fig. 1. Temperature-jump relaxation curves in 30 vol% ethyleneglycol/water mixtures of (a) deuterohemin without quinine, and (b) deuterohemin with 47 μM quinine. Conditions: 13.4 μM deuterohemin, pH 7.5, $T = 11.9$°C, 0.01 M phosphate, 0.1 M NaCl; temperature jump 4.3°C, wavelength 391 nm. The registrated optical extinction curve is superimposed to the fit curve with (a) one exponential, $\tau = 0.28$ ms, and (b) two exponentials, $\tau_1 = 0.343$ ms, $\tau_2 = 12.45$ ms.

Beckman Acta M6 spectrophotometer. For the relaxation kinetic measurements a conventional temperature jump equipment with optical detection and condenser discharge (50 nF) is operated at a voltage of 20 kV. The measuring cell, designed according to ref. [19] has a light path of 0.7 cm and an effective volume of 0.56 cm^3. The relaxation curves were recorded with a Datalab

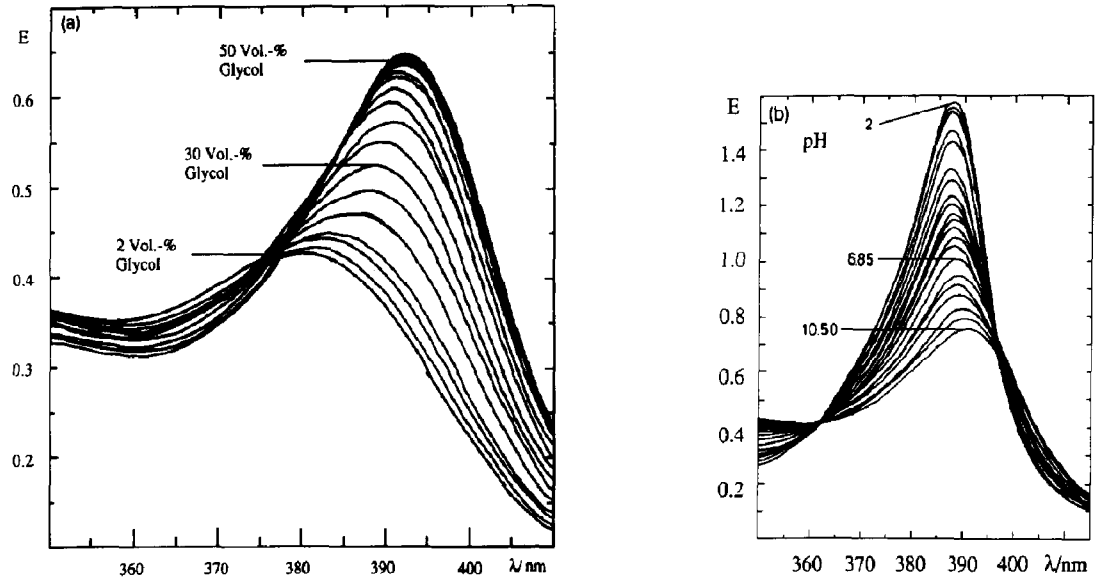

Fig. 2. Absorption spectra of deuterohemin (a) as a function of the ethyleneglycol content at pH 10.75, 8 μM heme, and (b) pH dependence at 50 vol% ethyleneglycol, 9.1 μM heme; 25°C, demonstrating the spectral changes with the formation of monomers at increasing glycol concentration and with the protonation of the monomers. The pH values for the curves in (b) read from above: ~ 2, ~ 1, 3.7, 3.9 5.0, 5.8, 5.9, 6.05, 6.2, 6.3, 6.4, 6.5, 6.65, 6,75, 6.85, 7.0, 7.15, 7.3, 7.6, 7.9, and 10.5.

DL 905 transient recorder and analyzed for up to three exponentials using the computer program DISCRETE [20].

3. Results and discussion

A typical kinetic progress curve after a temperature jump in the system deuterohemin/quinine is shown in Fig. 1. Three relaxation phases can clearly be distinguished: A fast jump is followed by a process in the range of about one millisecond and a still slower process in the range of 10 milliseconds. Experiments without quinine show unambiguously that the slowest relaxation in Fig. 1b has to be assigned to the interaction of deuterohemin with quinine. The faster processes which are also observed in the quinine free solution correspond to the dimerization of the hemins. Protonation steps of deuterohemin show up in the fast optical density jump.

Thus, quinine binding is a rather slow process, slower than the formation of μ-oxo bridges in the dimerization of the hemins and suggests that specific complexation with the iron is involved, as has been reported in literature [7,10,11]. Indeed, we find no such slow process for quinine binding to the iron free proto- and deuteroporphyrin (E. Volpert, unpublished results). However, the slow binding kinetics is also present in the reaction of deuterohemin with chloroquine, where specific interaction with the iron has not been found [11].

We will first discuss the results of the experiments without added quinine in order to characterize the kinetics of dimerization, which is important for understanding further interactions of the hemins. Details of the analysis [22] will be published separately.

3.1 Dimerization and protonation of deuterohemin

It is well established that Fe(III)-porphyrins dimerize both in the crystalline state [23,24] and in solution [25,26] by forming μ-oxo bridges. The reaction is pH dependent.

The spectral behaviour of deuterohemin in aqueous solutions containing ethyleneglycol is shown in Fig. 2. It manifests the changes in the Soret region on going from dimers at low glycol to monomers at high glycol concentration at alkaline pH (Fig. 2a) and on protonation of the hemin species, predominantly monomers at 50 vol% glycol (Fig. 2b). Monomers are characterized by sharper bands at longer wavelengths. A further test of the presence of monomers is the comparison of spectra when hemin is solubilized in sodium dodecylsulfate micells.

No detailed reaction scheme which would comprise the different hydrolytic equilibria and dimerization steps is discussed in the literature. Differently defined apparent dimerization constants are given which contain the proton concentration with powers of zero [15,27], one [13,28] and two [29,30]. Thus, comparison is hardly possible.

We have tried to analyze our data for deuterohemin dimerization in terms of a general mechanism which incorporates the pH-independent dimerization steps of protonated and deprotonated hemin monomers, $MH + MH \rightleftharpoons DH_2$ (equilibrium constant K_1), $M + MH \rightleftharpoons DH$ (K_2) and $M + M \rightleftharpoons D$ (K_3) and the hydrolytic equilibria for monomers and dimers, $MH_2 \rightleftharpoons MH + H$ (pK_{M1}), $MH \rightleftharpoons M + H$ (pK_{M2}), $DH_2 \rightleftharpoons DH + H$ (pK_{D1}), $DH \rightleftharpoons D + H$ (pK_{D2}). The monomeric species denote M^-: HO–Fe(P)–OH, MH: HO–Fe(P)–OH_2, MH_2^+: H_2 O–Fe(P)–OH_2, the dimeric species D^{2-}: HO–Fe(P)–O–Fe(P)–OH, etc. The reaction scheme is characterized by five independent equilibrium constants. Assumption that the two equivalent binding sites for the protons in the dimeric species are independent ($pK_{D1} = pK_{D2}$) reduces the number of thermodynamically independent reactions to four.

Taking pK_{M1}, pK_{M2}, pK_D and K_3 as the independent parameters, it is straightforward to calculate as a function of pH the dimerization constants K_1 and K_2 and the apparent dimerization constant which comprises the various protonated species of monomers and dimers respectively. From the reaction scheme it is obvious that at lower pH values the system is always shifted to the monomer side.

A consistent set of the four parameters discussed above can be obtained from relaxation kinetic measurements and spectrophotometric pH

titration. pK_{M1}, pK_{M2} and K_3 can be reliably determined. The pK_D of the dimers is determined with greater uncertainty. We consider it important to analyze both relaxation times and amplitudes, since their concentration dependence gives independent information on the binding constant. The consistency of the determined values is an important criterion for the validity of the assumed mechanism.

The procedure is as follows: The relaxation data at alkaline pH lead to the dimerization constant K_3. The relaxation curves at pH 11 are single exponentials with increasing optical extinction at 391 μm and almost no fast jump. Plots of the square of the reciprocal relaxation time vs. the total heme concentration show a linear dependence which is characteristic of a dimerization reaction [30], $(1/\tau)^2 = k_d^2 + 8k_d k_a C_{heme}$, where k_d, k_a denote the dissociation and association rate constants and C_{heme} the total hemin concentration in monomer units. From slope and intercept the value of K_3 can be deduced. K_3 can also be determined from the concentration dependence of the relaxation amplitudes since it is possible to localize the amplitude maximum in a plot of $\Delta E/C_{heme}$ vs. C_{heme}. We find consistency with the kinetically determined value.

In aqueous solutions without ethyleneglycol (EG), where predominantly dimers are present, the intercept in the kinetic plot is too small to be determined reliably and only the amplitude data allow an estimation of the equilibrium constant.

The protonation steps appear in the temperature jump experiments as a fast, not time resolved optical extinction change in the direction of decreasing optical extinction at 391 nm. Essential information can be obtained here from the amplitudes. The pH dependence of the amplitudes follows exactly the theoretical relation expected for a protolytic step in a buffered solution, exhibiting a maximum at $pH = pK$ ($\Delta E \sim c_H/K/(1 + c_H/K)^2$. pK-values of 7.0 can be deduced for the binding of the first proton (corresponding to pK_2) to the hydroxy form of hemin monomers, independent of the glycol content.

Spectrophotometric pH titration in the range from pH 11 to 3 allow the determination of the remaining two pK values, the binding of the second proton to the monomer, pK_{M1}, and the protonation of the dimer, pK_D, assumed to have independent binding sites. Literature data [13] for the extinction coefficients of the dimers are incorporated in the fit of the titration data. Another experimental quantity which contains information on the pK_D-value is the strong pH dependence of the relaxation amplitudes of the slow dimerization reaction, showing even a change in sign at about pH 6.7. Nevertheless, the pK_D value has the largest uncertainty. Table 1 lists the results.

We have also reevaluated the data given by Brown et al. [13] in a wide concentration and pH range for the aqueous solution (without glycol) in terms of the above mechanism and find an excellent fit of the data. The dimerization constant K_3 resulting from the reevaluation is about an order of magnitude smaller than our value. Problems of stability of the deuterohemin solution without added glycol may be responsible for some incon-

Table 1

Dimerization and hydrolytic equilibria of deuterohemin in water/ethyleneglycol mixtures

Constant	Ethyleneglycol (vol%)		
	0	30	50
Dimerization step: $2M^- \rightleftharpoons D^{2-}$: (M ≙ HO–FeP–OH)			
K_3/M^{-1}	2.5×10^{7} [a]	1.8×10^{5} [b]	1.9×10^{4} [b]
ΔH_3/kJ mol^{-1}			−36 [c]
$k_{ass3}/M^{-1}s^{-1}$	5.7×10^{8} [d]	9.8×10^{7} [b]	5.0×10^{7} [b]
k_{diss3}/s^{-1}	2.3×10^{1} [d]	5.6×10^{2} [b]	2.7×10^{3} [b]
Protolytic equilibria			
pK_{M1}	4.8 [e]	$\Delta H_{M1} = 0$ [f]	
pK_{M2}	6.9 [f]	$\Delta H_{M2} = 21$ [f] kJ/mol	
pK_D	≤ 6.7 [g]		

[a] From concentration dependence of slow phase relaxation amplitudes at pH 11.

[b] From concentration dependence of slow phase relaxation times at pH 11, yielding k_{ass} and k_{diss}.

[c] From slow phase relaxation amplitudes at pH ≥ 9.

[d] From concentration dependence of slow phase relaxation times, yielding (k_{ass} k_{diss}) together with K_3.

[e] From titration in 50%EG.

[f] From pH-dependence of fast-phase amplitudes in 30% and 50% EG and titration in 0% and 50% EG.

[g] From titration and pH dependence of slow-phase amplitudes in 50% EG.

sistencies and we were not able to reproduce the experimental data given in [13].

In summary, quantitative estimates in terms of a general reaction scheme of dimerization and hydrolysis equilibria have been obtained, which show the strong influence of ethyleneglycol on the extent of dimerization. The degree of dimerization (relative amount of heme incorporated in dimers) can be calculated with the equilibrium constants given above. This is best be done by introducing the apparent dimerization constant

$$K_{app} = \sum D/\left(\sum M\right)^2 = K_3(1 + c_H/K_D)^2/(1 + c_H/K_{M2} + c_H^2/K_{M1}K_{M2})^2 \quad (1)$$

where ΣD and ΣM denote the sum of the concentrations of the differently protonated monomer and dimer species. The values of the equilibrium constants are given in Table 1. The degree of dimerization β is given by the expression $1 - \beta = [(1 + 8K_{app}C_{heme})^{0.5} - 1]/4K_{app}C_{heme}$. It is practically constant in the pH range from 11 to 7, and amounts to about $\beta = 0.15$ at 50%EG, 0.5 at 30% EG and 0.9 at zero EG content. At lower pH values the amount of dimers decreases monotonically. In 30% and 50% EG we expect to have almost exclusively monomers at pH 4.

3.2 Quinine binding

3.2.1 Spectrophotometric titration

Spectral changes in the Soret range upon titration of a deuterohemin solution with quinine are shown in Fig. 3. The marked decrease of the extinction around 390 nm clearly demonstrates the interaction. No true isosbestic point in the range of 350 to 360 nm is observed. This might be an indication that more than one binding equilibrium is involved. Free quinine begins to absorb in that wavelength range, so that an exact isosbestic point is not expected under the conditions of Fig. 3. But also difference spectra against equimolar quinine solutions do not lead to a more precisely defined isosbestic point. Figure 4 shows plots of

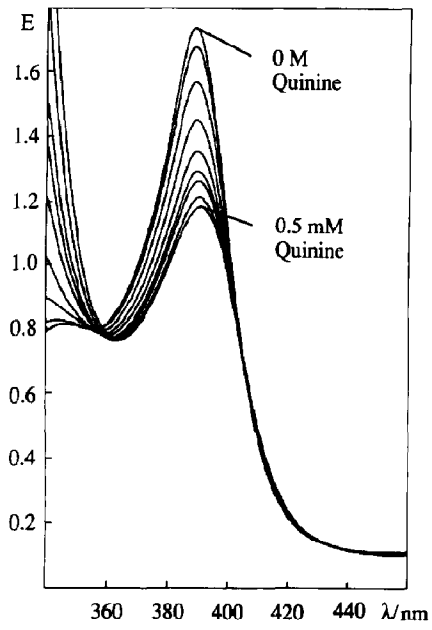

Fig. 3. Absorption spectra of deuterohemin in the presence of quinine in 50 vol% ethyleneglycol/water mixtures, pH 7.25, 19.4 μM heme, 22°C, 0.01 M Bis-Tris, 0.1 M NaCl, and light path 1 cm. The quinine concentrations read 0, 9.4, 36.2, 84.4, 152, 207, 253, 380, 506 μM.

the extinction at 392 nm vs. the total quinine concentration.

A rigorous analysis of these curves requires

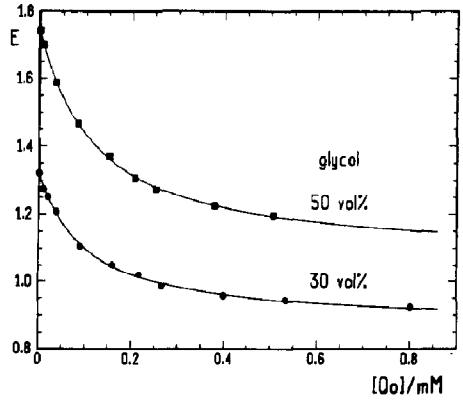

Fig. 4. Spectrophotometric titration of deuterohemin with quinine in ethyleneglycol/water mixtures, pH 7.25, wavelength 392 nm. For conditions see Fig. 3 Solid lines show fit of the data to a 1:1 binding reaction $E - E_0 = \Delta\epsilon[x - (x^2 - 4c_{Mo}c_{Qo})^{0.5}]$, where $x = c_{Mo} + c_{Qo} + 1/K$. c_{Mo}, c_{Qo} are total hemin and quinine concentration, K apparent binding constant; the values of K are given in Table 2.

Table 2

Quinine binding to deuterohemin in water/ethyleneglycol mixtures [a]

pH	Ethyleneglycol (vol%)	
	30	50
6.9	14.3×10^3	–
7.25	10.7×10^3	9.3×10^3
9.0	6.7×10^3	3.2×10^3

[a] Apparent binding constants K/M^{-1} obtained from spectrophotometric titration at 392 nm, analyzed in terms of a 1:1 complex formation. Conditions 22°C, 19 to 24 μM deuterohemin, buffer 0.01 M Bis-Tris (pH 6.9, 7.25), 0.01 M borate (pH 9)

the knowledge of many parameters—binding constants and extinction coefficients of monomers and dimers—which cannot be obtained by equilibrium data alone. Assuming the spectral changes to be proportional to the amount of complex formed, which requires that monomeric and dimeric hemin react equivalently, we find that the data can quite well be described by a 1:1 binding scheme. The values obtained by this procedure are apparent binding constants. They are listed in Table 2.

By comparing the data in Table 1 and 2 it is seen that drug binding and selfassociation to μ-oxo dimers is of comparable strength. The binding constants are, at least at neutral pH, only very slightly influenced by the ethyleneglycol content and thus by the amount of dimers present. Comparison with the value found for quinine binding to proto-hemin [32], $K = 11.3\times10^3\ M^{-1}$ (pH 9, 50 vol% EG, 24°C), shows that the vinyl groups, which have a strong effect on the dimerization, seem also to be involved in stabilizing the quinine – hemin complex.

3.2.2 *Relaxation kinetics*

The relaxation kinetic progress curves (see Fig. 1) are characterized by a fast, not time resolved optical density jump and two relaxation phases, well separated on the time axis. The fast processes have to be attributed to deuterohemin dimerization. The slow relaxation process corresponds to the binding of quinine. The increasing temperature shifts the binding equilibrium to the uncomplexed species with higher optical density. The measurements were done at a lower temperature in order to minimize cavitation effects.

The curve can be fitted with high accuracy by two exponentials in the whole concentration and pH range. At present we do not think that an additional faster quinine binding process is involved in the fast relaxation phase. This results from the following observation: The amplitude of the dimerization reaction changes sign when going from acid to alkaline pH. It passes zero at about pH 6.7. At this pH just a single exponential relaxation curve is observed.

Figure 5 shows the concentration dependence of the relaxation times and the relaxation amplitudes of the slow relaxation process. As the abscissa the sum of the free concentrations of quinine and hemin has been chosen, which is the relevant concentration parameter for a simple 1:1 binding reaction. The concentrations have been calculated with a binding constant given by the ratio of slope and intercept. The concentration of deuterohemin is under most conditions much smaller than the concentration of quinine so that the sum of the free concentrations is close to the weighed-in total concentration of quinine.

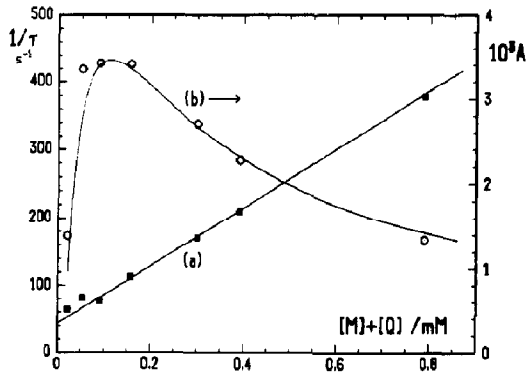

Fig. 5. Quinine binding to deuterohemin in 30 vol% ethyleneglycol/water mixtures, pH 7.5, 0.01 M phosphate, 0.1 M NaCl, 16.2°C; 13.4 μM heme. Abscissa: sum of free quinine and free deuterohemin concentration calculated with a binding constant of $10^4\ M^{-1}$ (see text); the total quinine concentration is given by 9.9, 47.1, 88.9, 160, 308, 400, 800 μM. Left ordinate: (a) reciprocal relaxation times; right ordinate: (b) relaxation amplitudes (optical extinction change). Solid lines: fit of the data to a 1:1 binding reaction (see text).

The concentration dependence is as expected for a binding reaction: The reciprocal relaxation times increase linearly with the sum of the free concentrations and the amplitude curve exhibits a maximum near a concentration equal to the reciprocal binding constant. We find it very important—as in the case of the dimerization reaction —to analyze both relaxation times and amplitudes, since the two sets of data contain independent information on the binding. From kinetics the binding constant is obtained from the ratio of slope and intercept, in the amplitude curve the binding constant determines the form of the bell shaped curve and the position of the maximum.

The theoretical curves in Fig. 5 have been calculated on the basis of a 1 : 1 binding reaction. The reciprocal relaxation times are described by

$$1/\tau = k_D + k_R(c_M + c_Q) \qquad (2)$$

leading to a dissociation rate constant of 44 s^{-1} and a recombination rate constant of 4.4×10^5 M^{-1} s^{-1}. The relaxation amplitudes, i.e. the optical extinction changes δE for a binding reaction are given by $\delta E = \Delta\epsilon\, d\Gamma\, \delta \ln K$, where $\Delta\epsilon$, d, $\delta \ln K$ denote the molar extinction change, light path, and relative change of the equilibrium constant due to the temperature jump.

The transfer function is given by

$$\Gamma^{-1} = \left(c_M^{-1} + c_Q^{-1} + c_{MQ}^{-1}\right)^{-1} = C_{heme} K c_Q / \left[(1 + Kc_Q)^2 + KC_{heme}\right] \qquad (3)$$

As in the static measurements the concentration of quinine is in excess, so that the free concentration of quinine is close to the total concentration.

The amplitude curve in Fig. 5 has been calculated with the kinetically determined binding constant $K = 10$ $(mM)^{-1}$. As is seen there is a satisfactory description of the rate and amplitude data. It appears that a simple 1 : 1 binding scheme can quite well describe both the static binding and the kinetics of the slow relaxation process. However, the implicit assumption of equal reactivity of hemin monomers and hemin incorporated in dimers is not fully consistent with the observed fast relaxation process and the analysis given so far should be taken as a first approximation: The relaxation times and amplitudes of the fast dimerization step should remain unchanged upon addition of quinine, if the assumption that binding is independent of the state of hemin aggregation were true. However, a discernible decrease in the amplitudes of the fast process appears, indicating that dimerization is different for uncomplexed and complexed hemins. A more detailed reaction mechanism has to be considered. We have considered the limiting case that deuterohemin dimers do not bind quinine. The relaxation times are in this case given by the expression

$$1/\tau = k_D + k_R[c_M + c_Q/(1 + 4K_D c_M)]. \qquad (4)$$

The time constants can reasonably well be described with this equation, leading to somewhat larger values for the rate constants, but in numerical calculations of the relaxation spectrum, using the program given in ref. [21], we find so far no consistent description of both relaxation times and amplitudes.

The simplicity of the relaxation behavior seems not to require the introduction of additional reaction steps necessary to account for the postulated stoichiometry of two hemins to one quinine [2,10]. In this case we would expect a more complicated relaxation spectrum. Two limiting cases can be discussed: If there are two independent binding sites with different properties, we expect to find two relaxation times. In the case of a highly cooperative binding with respect to hemin, where the concentration of singly liganded quinine is negligible, the reaction is approximated by $2M + Q = M_2Q$ and we would not expect a linear dependence in the plot of Fig. 5.

3.2.3 pH dependence

The binding reaction is pH dependent. While the static binding constants do not very strongly depend on pH, the reciprocal relaxation times increase strongly with decreasing pH, as is seen from Fig. 6 for 30 vol% ethyleneglycol. A similar pH dependence is also found at 50% glycol (S. Kuhn, unpublished results), where there are predominantly hemin monomers in solution. The pH-dependence is characteristic for the binding

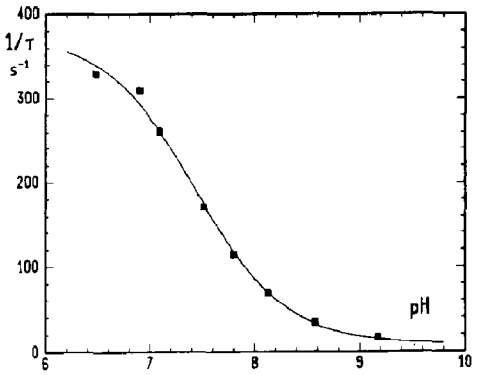

Fig. 6. Quinine binding to deuterohemin in 30 vol% ethyleneglycol/water mixtures. pH dependence of the relaxation times; 13.4 μM heme, 300 μM quinine, other conditions as in Fig. 5. Solid line: see text.

of a ligand to two substrate forms, a deprotonated and a protonated form, which rapidly interconvert. The acid form of the substrate reacts much faster than the alkaline form. The fit of the data to a function $1/\tau - (1/\tau)_{alk} \sim c_H/K/(1 + c_H/K)$ leads to an apparent pK of 7.4. The pK values associated with the deuterohemin molecule are definitely smaller than this value, the amplitude maximum of the fast jump is found at pH 7 (see Table 1) both in hemin solutions with and without quinine. The pH-dependence might arise from the protonation of the chinuclidin-nitrogen, which leads to an enhanced electrostatic interaction with the negatively charged deuterohemin molecule. Different reactivities of monomers and dimers could also contribute.

We expect to clarify the various open questions by studying the kinetics of the interaction using different hemins, and different quinoline containing antimalarial drugs. Comparison of proto- and deuterohemin will show the influence of the vinyl groups which strongly determine the dimerization behaviour. Molecules as Fe-TPPS (tetraphenylporphinsulfonic acid) which does also dimerize, should give information on the charges involved. Comparison of quinine and chloroquine will add information on the proposed importance [10] of the hydroxyl-group in the 9-position of quinine. We have especially stressed the use of ethyleneglycol–water mixtures, where the extent of dimerization of the hemins can be controlled and we expect further information by a detailed comparison of the relaxation kinetic behaviour at various glycol contents.

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