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A Study of the Properties of Hybrids of Oxyhaemoglobin and Deoxyhaemoglobin with Two Porphyringlobin Species

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The fluorescence of porphyringlobin is quenched on adding haemoglobin to its solutions. It is suggested that this result indicates the formation of hybrids (comprising a dimer of porphyringlobin and a dimer of haemoglobin) in which quenching occurs by energy transfer from the porphyrin to the haem groups of the protein. From an analysis of fluorescence quenching, dissociation constants were calculated for the hybrids of oxy- and deoxyhaemoglobin with the fast- and slow-moving porphyringlobin species isolated by chromatography on CM-Sephadex (Treffry & Ainsworth, 1974). The values obtained are: deoxyhaemoglobin–fast-moving porphyringlobin, 0.8×10^{-9}M; deoxyhaemoglobin–slow-moving porphyringlobin, 5×10^{-10}M; oxyhaemoglobin–fast-moving porphyringlobin, 0.8×10^{-6}M; oxyhaemoglobin–slow-moving porphyringlobin, 1.2×10^{-7}M. The rates of reactions of solutions of haemoglobin and porphyringlobin, containing hybrids, with the thiol reagent 4,4′-dithiodipyridine showed that the thiol groups of the hybrids deoxyhaemoglobin–fast-moving porphyringlobin and oxyhaemoglobin–slow-moving porphyringlobin react more slowly than expected on the basis of composition alone: this result indicates that the deoxy and slow-moving conformations are the more stable, imposing themselves partially on to the fast-moving or oxy dimer of the hybrid. Also the rate of the reaction of CO with deoxyhaemoglobin is decreased when slow-moving porphyringlobin is added to its solutions: this is reflected in a movement of the oxygen equilibrium curve of such a mixture to higher oxygen partial pressures. Similar experiments with deoxyhaemoglobin solutions containing fast-moving porphyringlobin, showed an initial increase in the rate of CO uptake. Correspondingly, the oxygen equilibrium curve of the mixture showed an increased affinity for oxygen. Approximate calculations to determine the oxygen equilibria of the hybrids indicate that a functional dimer retains co-operative characteristics even when the dimer accompanying it within the tetramer has the reacted conformation.

In two preceding papers (Treffry & Ainsworth, 1974; Ainsworth & Treffry, 1974) we have shown that porphyringlobin, prepared by adding excess of protoporphyrin IX to globin, is heterogeneous, but that the two main constituents, termed fast-moving and slow-moving, may be isolated by chromatography on CM-Sephadex. We have also shown that fast- and slow-moving porphyringlobin have properties which are similar, in some respects, to oxy- and deoxyhaemoglobin respectively, that the slow-moving conformation is the more stable and that hybrids formed between fast- and slow-moving porphyringlobin have properties indicating that the slow-moving conformation has a greater influence on the fast-moving conformation than the reverse.

In the present paper we describe a study of mixtures of the two porphyringlobin species with oxy- and deoxy-haemoglobin. We show that hybrids are formed in these solutions and that conformational change is transmitted between the porphyrin and haem dimer of the hybrid.

Experimental

Materials and methods

The materials used and methods employed were as described by Treffry & Ainsworth (1974) or Ainsworth & Treffry (1974).

Additional methods

Oxygen equilibria. The equilibrium of oxygen and haemoglobin in solution alone and in admixture with fast- and slow-moving porphyringlobin was determined by the method of Imai *et al.* (1970). The required buffer solution was contained in a thermostatically controlled 10mm glass cuvette which was stirred with a magnetic flea and de-aerated by a stream of N_2. After complete de-aeration was achieved, as detected by an oxygen electrode (Medical Workshop, University of Bristol) inserted in the side of the cuvette, concentrated samples of the proteins to be examined were introduced: a further short

period of bubbling N_2 removed the oxygen bound to the haemoglobin sample. The cuvette assembly, incorporating the oxygen electrode, was placed in a Cary 14 recording spectrophotometer and the change in pigment composition consequent on the introduction of oxygen monitored continuously by absorbance measurements at 578nm. The rate of oxygen addition was controlled by introducing bubbles of air into the cuvette by a variable speed peristaltic pump (12000 Varioperpex; LKB Produkter AB, Bromma, Sweden); the resulting oxygen concentration was measured continuously with the oxygen electrode. Complete oxygenation generally took 30min. The spectrum of the pigments in the Soret region were measured before and after oxygenation to confirm that methaemoglobin formation had not occurred. The oxygen electrode was calibrated by using solutions equilibrated with air and with oxygen-free N_2; a response identical with the latter was obtained with solutions containing sodium dithionite.

Fluorescence measurements. The method of Ainsworth & Treffry (1974) was employed with the following modification. The fluorescent sample was contained in a 1mm silica cuvette oriented at 45° to the incident light, and the fluorescence observed at 90° to the exciting light and at 180° to the direction of reflexion of exciting light from the cuvette surfaces.

Quenching of porphyringlobin fluorescence by haemoglobin. Oxyhaemoglobin (approx. 0.1mM in 0.1M-potassium phosphate buffer, pH7.0) was added by an Agla micrometer syringe to 1.5ml of porphyringlobin solution (approx. 1μM in 0.1M-potassium phosphate buffer, pH7.0) contained in a vial. After incubation for 15min at 20°C, the fluorescence intensity of a sample was measured. For experiments with deoxyhaemoglobin, a minute amount of sodium dithionite was added to deoxygenate the solution. The total absorbance of these solutions at 403nm never exceeded 0.08 units (1mm cell). The observed fluorescence was corrected for the loss of exciting light by haemoglobin absorption and for the dilution of the porphyringlobin sample by multiplication by the factor $(1-e^{-E_1})E_3/(1-e^{-E_3})E_2$, where E_1 is the absorbance of the stock solution of porphyringlobin, E_2 the absorbance of porphyringlobin in the mixture and E_3 the total absorbance of the mixture. With the solutions used, no correction for the reabsorption of fluorescence was required. The fluorescence of the stock solution of porphyringlobin was taken as 100 units and all other measurements adjusted relative to it. No significant photodecomposition took place during the course of these measurements.

Stopped-flow determinations. The reaction of CO with haemoglobin, alone and in admixture with porphyringlobin, was monitored, by using a stopped-flow apparatus constructed by Ainsworth & Milnes, by the absorption of light at 437.5nm. The bandwidth of the light was 1.65nm.

Emission spectrum. The emission spectrum of porphyringlobin was recorded by using the instrument of Ainsworth & Winter (1964) and corrected for plotting on a wavenumber axis as described by Parker & Rees (1960). The vertical axis was adjusted (to calculate the overlap integral) by equating the peak emission intensity with the extinction coefficient of the peak of the absorption band of least frequency. The peak of the emission band occurred at 15955 wavenumbers.

Results

Quenching of porphyringlobin fluorescence by haemoglobin

The fluorescence of porphyringlobin is immediately quenched on adding either oxy- or deoxyhaemoglobin to its solutions. This observation can be explained by assuming that tetrameric hybrids are formed from $\alpha\beta$ dimers of the two pigments (Bunn & Jandl, 1968; Benesch *et al.*, 1966*a*,*b*), and that quenching arises by the transfer of excitation energy from the porphyrin to the haem groups. With this model it is possible to estimate the dissociation constant for the hybrid, if the dissociation constants of the two pure species are known. Defining the dimer–tetramer equilibrium of haemoglobin by the constant K_H, we have:

$$K_H = [H]^2/[H_2] \quad (1)$$

where [H] is the concentration of haemoglobin dimers and $[H_2]$ the concentration of tetramers. The value of K_H, so defined, is identical with values (quoted in the literature) where the concentrations of all the species in the equilibrium are given in monomeric units. Rearranging eqn. (1), we obtain:

$$[H] = [-K_H + (K_H^2 + 8[H_0]K_H)^{\frac{1}{2}}]/4 \quad (2)$$

where $[H_0] = [H]+2[H_2]$.

Eqn. 2 may now be modified to represent the situation arising when porphyringlobin is also present, i.e.:

$$[H] = \langle -K_H + \{K_H^2 + 8K_H([H_t] - [HP])\}^{\frac{1}{2}} \rangle /4 \quad (3)$$

where [HP] is the concentration of haemoglobin dimers in hybrid tetramers; thus the total concentration of haemoglobin dimers is $[H_t] = [H_0]+[HP]$.

Defining similar relationships for porphyringlobin (P), and a dissociation constant for the hybrid equilibrium:

$$K_M = \frac{[H][P]}{[HP]} \quad (4)$$

we obtain:

$$x = \frac{1}{16m}\{-h + (h^2 + 8h - 8hrx)^{\frac{1}{2}}\} \{-p + (p^2 + 8p - 8px)^{\frac{1}{2}}\} \quad (5)$$

where $x = [HP]/[P_t]$; $h = K_H/[H_t]$; $p = K_p/[P_t]$; $r = [P_t]/[H_t]$ and $m = K_M/[H_t]$.

Values of x were obtained for known values of h, p and r and assumed values of K_M by iteration according to Newton's method (Uspensky, 1948):

$$x_{n+1} = x_n - \frac{f(x)}{f'(x)} \text{ and } f(x) = 0$$

in which the initial estimate of x was chosen to give a positive value to both square-root terms of eqn. (5).

K_M can now be estimated by comparing calculated values of x with experimental values of x. The latter are obtained from the relationship $x = \Delta F/\Delta F_0$ where ΔF is the decrease in fluorescence observed on adding haemoglobin to porphyringlobin and ΔF_0 is the corresponding decrease observed when sufficient haemoglobin is added to convert all the porphyringlobin present into the hybrid form. Unfortunately, ΔF_0 is not obtainable directly, but an estimate can be obtained by extrapolation. We have, from eqn. (4):

$$\frac{[HP]}{[HP]+[P]} = \frac{[H]}{K_M+[H]} \quad (6)$$

Now, eqn. 2 shows that [P] increases with $[P_0]$, and eqn. 3 shows that [H] increases with $[H_t]$: we can therefore modify eqn. (6) to obtain the following approximations:

$$x = \frac{[HP]}{[P_t]} = \frac{\Delta F}{\Delta F_0} \approx \frac{[H_t]}{K_M+[H_t]}$$

or

$$\frac{1}{\Delta F} \approx \frac{K_M}{\Delta F_0} \cdot \frac{1}{[H_t]} + \frac{1}{\Delta F_0} \quad (7)$$

Plots of ΔF^{-1} as a function of $[H_t]^{-1}$ are almost linear, as required by eqn. (7). Taking the fluorescence of porphyringlobin as 100 units (the fluorescence of both fast- and slow-moving species is the same for equal amounts) the values of ΔF_0 obtained by extrapolation are: slow-moving porphyringlobin–oxyhaemoglobin, 20; slow-moving porphyringlobin–deoxyhaemoglobin, fast-moving porphyringlobin–oxyhaemoglobin and fast-moving porphyringlobin–deoxyhaemoglobin, 37. The extrapolation of the plot for slow-moving porphyringlobin–deoxyhaemoglobin is rather less certain than the others because of the plot's greater curvature; thus a linear extrapolation weighting the higher values of $[H_t]$ more heavily gives $\Delta F_0 = 33.3$, whereas an extrapolation that attempted to continue the curve gave the value quoted above. The linearity of the plots and the nature of the extrapolations may be judged from Fig. 1 where x^{-1} is plotted as a function of $[H_t]^{-1}$ by using the extrapolated values of ΔF_0 that have been given. Assuming the following dissociation constants: fast-moving porphyringlobin, 2.8×10^{-6}M; slow-moving porphyringlobin, 8×10^{-8}M (Ainsworth & Treffry, 1974); oxyhaemoglobin, 2.9×10^{-6}M (Kellett, 1971*a*); deoxyhaemoglobin, 3×10^{-12}M (Thomas & Edelstein, 1972), eqn. (5) gave the calculated relationships $x^{-1} = f([H_t]^{-1})$ plotted (for comparison) in Figs. 1(*a*)–1(*d*). The fits to the experimental values of x^{-1}, also shown in Figs. 1(*a*)–1(*d*), were obtained by using values interpolated from calculated relationships $x = f(K_M)$ for $[H_t] = 0.5$, 1.0, 2.0 and 3.0μM (dimers). On the basis of these fits, the following dissociation constants for the hybrids, K_M, were obtained: slow-moving porphyringlobin–oxyhaemoglobin, $1.2\times10^{-7}\pm0.2\times10^{-7}$M; slow-moving porphyringlobin–deoxyhaemoglobin, $5.0\times10^{-10}\pm0.05\times10^{-10}$M (for $\Delta F_0 = 37$, this value of K_M is employed in later calculations) and $4.2\times10^{-10}\pm0.05\times10^{-10}$M (for $\Delta F_0 = 33.3$); fast-moving porphyringlobin–oxyhaemoglobin, $8.0\times10^{-7}\pm0.4\times10^{-7}$M; fast-moving porphyringlobin–deoxyhaemoglobin, $8\times10^{-10}\pm1\times10^{-10}$M. Note that these values indicate that fast-moving porphyringlobin–oxyhaemoglobin is the only hybrid more stable than both pure species contributing to its formation; in every other instance the stability of the hybrid lies between that of each of its parent compounds. The error attached to the dissociation constants represents the average of the difference observed in each instance, between the values of K_M predicted by $x = f(K_M)$ at each $[H_t]$ and the value of K_M chosen to provide the best fit for all four values of $[H_t]$ considered together.

Thiol group reactivity of mixtures of porphyringlobin and haemoglobin

We have shown (Ainsworth & Treffry, 1974) that the thiol groups of fast-moving porphyringlobin react with 4,4′-dithiodipyridine (4-Py-S-S-4-Py) at the same rate as those of oxyhaemoglobin, and that this rate is greater than the rate at which both slow-moving porphyringlobin and deoxyhaemoglobin react with the thiol reagent. In principle, however, hybrid formation can only be detected on the basis of thiol group reactivity, if the initially different reactivity of the pure species is modified on forming the hybrid. Fig. 2 shows progress curves for the reaction of oxyhaemoglobin and slow-moving porphyringlobin, both alone and in admixture, with 4-Py-S-S-4-Py present in large excess. Fig. 2 also shows calculated curves, the first of which is based on the assumption that the two pigments present in the mixture react independently with 4-Py-S-S-4-Py irrespective of whether their dimeric subunits are present in the hybrid or not. It will be seen that this curve does not correspond to the experimental curve for the mixture of the two pigments. However, on the basis of the dissociation constants given in the preceding section we calculate that 47.6% of the oxyhaemoglobin is present in the hybrid; assuming that it reacts with 4-Py-S-S-4-Py at the rate of slow-moving porphyringlobin allows the calculation of a second curve that corresponds very closely to the experimental progress curve of the mixture. Similar

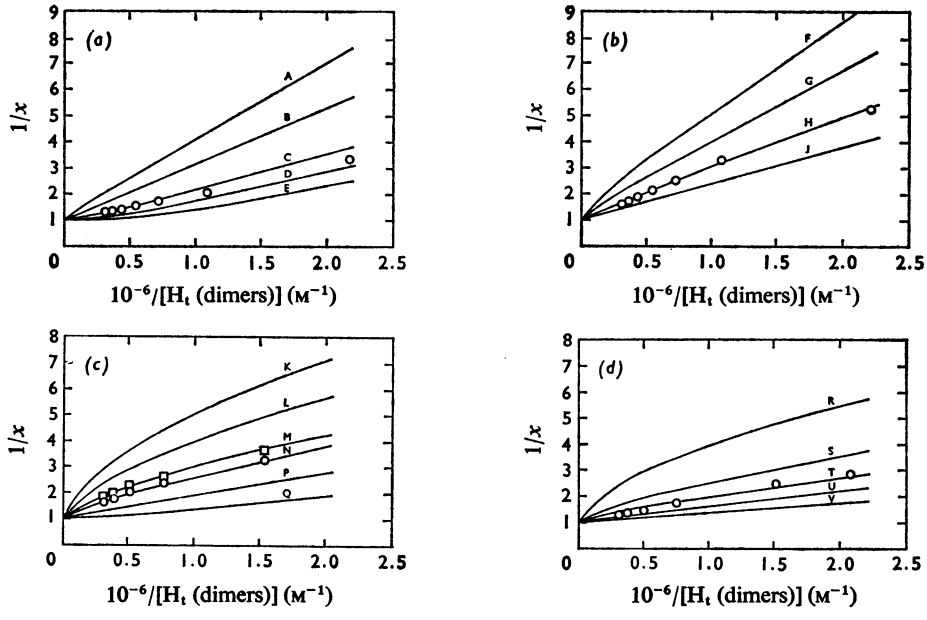


Fig. 1. *Quenching by haemoglobin of the fluorescence of porphyringlobin in* 0.1 M*-potassium phosphate buffer, pH* 7.0, 20°C, *excited at* 403 *nm*

The ordinate is defined in the text. (*a*) Oxyhaemoglobin–slow-moving porphyringlobin ([P_t] = 0.9066 μM) K_M: curve A, 3.75×10^{-7} M; curve B, 2.50×10^{-7} M; curve C, 1.20×10^{-7} M; curve D, 0.75×10^{-7} M; curve E, 0.375×10^{-7} M. (*b*) Oxyhaemoglobin–fast-moving porphyringlobin ([P_t] = 0.9066 μM) K_M: curve F, 1.75×10^{-6} M; curve G, 1.25×10^{-6} M; curve H, 0.80×10^{-6} M; curve J, 0.50×10^{-6} M. (*c*) Deoxyhaemoglobin–slow-moving porphyringlobin ([P_t] = 0.7969 μM) K_M: curve K, 1.00×10^{-9} M; curve L, 7.50×10^{-10} M; curve M, 5.00×10^{-10} M; curve N, 4.20×10^{-10} M; curve P, 2.50×10^{-10} M; curve Q, 1.00×10^{-10} M. (*d*) Deoxyhaemoglobin–fast-moving porphyringlobin ([P_t] = 0.7556 μM) K_M: curve R, 2.50×10^{-9} M; curve S, 1.25×10^{-9} M; curve T, 0.80×10^{-9} M; curve U, 0.50×10^{-9} M; curve V, 0.25×10^{-9} M. The concentrations of porphyringlobin and haemoglobin are given in dimer units. Each point is the mean of three determinations.

data and calculations are provided in Fig. 3 for the reactions of fast-moving porphyringlobin and deoxyhaemoglobin, both alone and when mixed. In this case, it is clear that, although the mixture reacts more slowly with 4-Py-S-S-4-Py than is indicated by its content of the pure species, it does not react as slowly as would be required if the hybrid reacted, as a whole, with the rate of deoxyhaemoglobin.

These observations indicate that hybrid formation occurs and that conformational change takes place in the hybrid, resulting in the hybrid moving towards the conformation of slow-moving porphyringlobin or deoxyhaemoglobin. This change appears to go further in the slow-moving porphyringlobin–oxyhaemoglobin hybrid than it does in the fast-moving porphyringlobin–deoxyhaemoglobin hybrid; probably because as was shown by Treffry & Ainsworth (1974), the rate at which fast-moving porphyringlobin is converted into slow-moving porphyringlobin is very much slower than the rate of conformational change in haemoglobin (Antonini & Brunori, 1969). The results further confirm the observation of Ainsworth & Treffry (1974) that hybrids formed between fast- and slow-moving porphyringlobin undergo a conformational change towards the conformation of the more-stable slow-moving species. Comparable observations have been made by Nagel & Gibson (1972), who showed that the conformation of deoxygenated $\alpha_2\beta_2^{3+}$ and $\alpha_2^{3+}\beta_2$ resembled that of deoxyhaemoglobin, and by Nagel & Bookchin (1973), who found the same result with $\alpha_2^{CN}\beta_2$ and $\alpha_2\beta_2^{CN}$.

Relative quantum yields of porphyringlobin–haemoglobin hybrids

In the penultimate section, values of ΔF_0 were obtained for each hybrid species. These values will now be considered in the context of the probability of energy transfer in each species.

The processes involved in the absorption and dissipation of light energy by porphyringlobin hybrids are summarized in Scheme 1. In this scheme, α and β

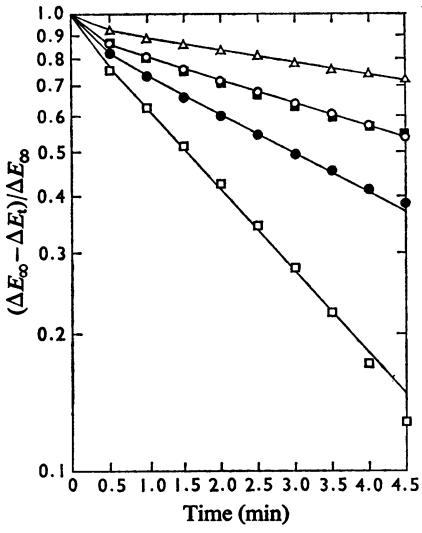


Fig. 2. *Time-course of reaction of* 4-*Py*-*S*-*S*-4-*Py* (0.440 mM) *with slow-moving porphyringlobin* (△; 3.94 μM *dimers*), *oxyhaemoglobin* (□; 5.31 μM *dimers*) *and a mixture of slow-moving porphyringlobin* (3.94 μM *dimers*) *with oxyhaemoglobin* (5.31 μM *dimers*) (○), *in* 0.1 M-*potassium phosphate buffer*, *pH* 7.0, 25°C

The reaction was monitored spectrophotometrically at 324 nm. ● represents the weighted average of △ and □. ■ is a weighted average of △ and □ assuming that the hybrid HP (2.53 μM dimers) reacts as slow-moving porphyringlobin.

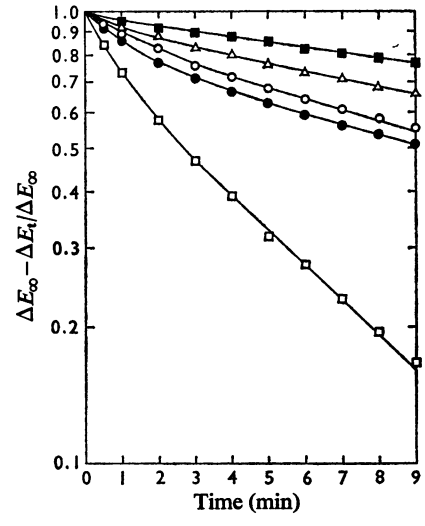


Fig. 3. *Time-course of reaction of* 4-*Py*-*S*-*S*-4-*Py* (0.233 mM) *with fast-moving porphyringlobin* (□; 4.30 μM *dimers*); *deoxyhaemoglobin* (■; 5.51 μM *dimers*) *and a mixture of fast-moving porphyringlobin* (4.30 μM *dimers*) *and deoxyhaemoglobin* (5.51 μM *dimers*) (○) *in* 0.1 M-*potassium phosphate buffer*, *pH* 7.0, 25°C

The reaction was monitored spectrophotometrically at 324 nm. ● represents the weighted average of □ and ■. △ is a weighted average of □ and ■ assuming that the hybrid HP (2.59 μM dimers) reacts as deoxyhaemoglobin.

represent the photoexcited porphyrin residues on the α and β chains. We assume that they absorb light equally, i.e. $I_\alpha = I_\beta$, and that the intrinsic rates of fluorescence (k_f) and radiationless deactivation (k_i) are the same. The rate of energy transfer between the groups is represented by k_1, and k_2 and k_3 sum the rate constants for energy transfer to the haem groups of the hybrid from the α and β subunits respectively. Further, assuming that the concentrations of excited α and β porphyrins are in a steady state, we obtain:

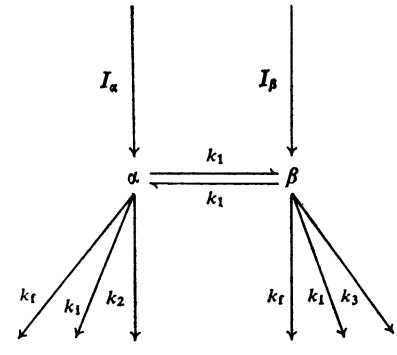
$$I\alpha = (k_1 + k_f + k_i + k_2)\alpha - k_1\beta$$
$$I_\beta = (k_1 + k_f + k_i + k_3)\beta - k_1\alpha \qquad (8)$$

whence:

$$\frac{\alpha}{\beta} = \frac{2k_1 + k_f + k_i + k_3}{2k_1 + k_f + k_i + k_2}$$

Taking the quantum yield of fluorescence (q) as the quotient of the rate of light emission and the rate of light absorption we obtain:

$$\frac{1}{q} = \frac{1}{q_0} + \frac{k_2}{k_f} w_\alpha + \frac{k_3}{k_f} w_\beta \qquad (9)$$



Scheme 1. *Processes involved in the absorption and dissipation of light energy by porphyringlobin hybrids*

For details see the text.

where $q_0 = k_f/(k_i + k_f)$, that is, the quantum yield in the absence of transfer. $w_\alpha = \alpha/(\alpha+\beta)$ and $w_\beta = 1 - w_\alpha$. Writing $w_\alpha = 0.5 + \Delta$, we have:

$$\frac{1}{q} - \frac{1}{q_0} = \frac{k_2 + k_3}{2k_f} + \frac{\Delta(k_2 - k_3)}{k_f} \qquad (10)$$

The second term may be regarded as the error incurred in making the simplifying assumption that $w_\alpha \equiv w_\beta$. Now $\Delta = 0.5\ (w_\alpha - w_\beta)$, therefore from eqn. 8 the maximum error (E) expressed as a fraction of $(k_2+k_3)/2k_f$ is:

$$E \nearrow \left(\frac{k_2-k_3}{k_2+k_3}\right)^2 \qquad (11)$$

k_2 and k_3 may be identified by assuming that the hybrids are formed from $\alpha_1\beta_1$ dimers (Rosemeyer & Huehns, 1967; Perutz *et al.*, 1968); that is, $k_2 = k_{\alpha_1\beta_2} + k_{\alpha_1\alpha_2}$; $k_3 = k_{\beta_1\alpha_2} + k_{\beta_1\beta_2}$ and hence $k_2+k_3 = 2k_{\alpha_1\beta_2} + k_{\alpha_1\alpha_2} + k_{\beta_1\beta_2}$, where the subscripts represent the subunits between which transfer is occurring.

According to Förster (1960), the rate constant for transfer is proportional to ϕJ. J is the overlap integral given by $\int_0^\infty \epsilon_A(\nu)\epsilon_D(\nu)d\nu$: in this expression $\epsilon_A(\nu)$ represents the absorption spectrum of haemoglobin and $\epsilon_D(\nu)$, the corrected emission spectrum of porphyringlobin normalized, at the peak, with the absorption band of least frequency. Both spectra are plotted on a wavenumber axis. ϕ is the quotient K^2/R^6. R is the distance between the donor and acceptor molecules and, in our calculations, it has been assumed to correspond to the Fe–Fe distances of horse haemoglobin. K is defined by:

$$K = \cos\psi_{DA} - 3\cos\psi_D \cdot \cos\psi_A$$

where ψ_{DA} is the angle between the transition moment vectors of both molecules and ψ_D and ψ_A are the angles between these respective vectors and the direction D → A. Spectroscopic studies by Gurinovich *et al.* (1961), Gouterman & Stryer (1962) and Anex & Umans (1964) have established that the last band of porphyrin is 100% polarized in the plane of the molecule, but its direction within the plane remains in some doubt (Corwin *et al.*, 1968).

We have therefore calculated the values of ϕ that arise when an angle θ, between 0–180°, is fixed between the transition moment vector and a line passing through the α and γ C atoms of the tetrapyrrole ring, assuming that the relative orientation of the rings for a given transfer is either that of horse oxy- or deoxyhaemoglobin and that the transition moment vector has the same direction in both donor and acceptor. These calculations show that the most important transfer for all θ is $\alpha_1\beta_2$, and that its relative importance increases markedly with θ. Examples of these calculations are given in Table 1.

Table 2 gives values of E:

$$E \nearrow \left(\frac{\phi_{\alpha_1\alpha_2} - \phi_{\beta_1\beta_2}}{2\phi_{\alpha_1\beta_2} + \phi_{\alpha_1\alpha_2} + \phi_{\beta_1\beta_2}}\right)^2$$

and

$$\frac{\Sigma\phi_D}{\Sigma\phi_O} = \frac{(2\phi_{\alpha_1\beta_2} + \phi_{\alpha_1\alpha_2} + \phi_{\beta_1\beta_2})_{deoxy}}{(2\phi_{\alpha_1\beta_2} + \phi_{\alpha_1\alpha_2} + \phi_{\beta_1\beta_2})_{oxy}}$$

It is evident that E is negligible throughout and that a comparison of the quantum yields of two porphyrin-globin hybrids may therefore be based on the first term of eqn. (10), thus:

$$\left(\frac{\Delta F_0}{F_0 - \Delta F_0}\right)_A \Big/ \left(\frac{\Delta F_0}{F_0 - \Delta F_0}\right)_B \equiv \frac{F_A}{F_B} = \frac{J_A \Sigma\phi_A}{J_B \Sigma\phi_B}$$

Table 2 shows that the structural factor $\Sigma\phi_D/\Sigma\phi_O$ must lie between 1.08 and 1.30 and therefore is much less important in determining relative quantum yields than the change in overlap integral $J_D/J_O = 2.49$. This stems directly from the dominance of the $\alpha_1\beta_2$ transfer and its comparative insensitivity to the change in haemoglobin structure.

Table 3 provides values of the ratio $F_A J_B / F_B J_A$ for six possible comparisons of the quantum yields of fluorescence. Referring to Table 3; ratio 1 suggests that fast- and slow-moving porphyringlobin hybrids with deoxyhaemoglobin have similar structures as far as transfer is concerned: the thiol group reactivity of the compounds (Fig. 3) confirms that the fast-moving porphyringlobin component of the hybrid is changed but to what extent is not known. Ratio 3 suggests either that the conformations of the two hybrids compared are different, in which event θ is approx. 30–40°; or if they are the same, that slow-moving

Table 1. *Values of ϕ for transfers in the oxy and deoxy conformations of horse haemoglobin as a function of θ*

R is employed with units of 10^{-8} cm with the following values: oxy $\beta_1\beta_2$, 34.1168; $\alpha_1\alpha_2$, 35.6492; $\alpha_1\beta_2$, 25.9457; $\alpha_1\beta_1$, 35.2087; deoxy $\beta_1\beta_2$, 39.0119; $\alpha_1\alpha_2$, 34.5727; $\alpha_1\beta_2$, 24.6802; $\alpha_1\beta_1$, 36.4561.

θ (°)	$10^{11}\phi$ for oxy conformation				$10^{11}\phi$ for deoxy conformation			
	$\beta_1\beta_2$	$\beta_1\alpha_1$	$\alpha_1\beta_2$	$\alpha_1\alpha_2$	$\beta_1\beta_2$	$\beta_1\alpha_1$	$\alpha_1\beta_2$	$\alpha_1\alpha_2$
0	106.0	179.5	342.0	98.62	60.15	137.0	422.3	118.4
30	139.2	157.1	596.8	109.5	65.63	134.9	670.5	153.9
60	59.70	16.10	939.9	51.21	32.87	17.43	1046	75.76
90	4.632	13.26	957.9	10.96	9.120	10.08	1119	12.09
120	0.4208	7.708	625.7	7.703	7.154	9.514	790.0	3.818
150	22.28	32.99	352.9	37.03	25.43	18.96	469.0	32.00

Table 2. *Values of* $\Sigma\phi_D/\Sigma\phi_O$, E_D *and* E_O *as a function of* θ

The table also gives values for J. For details see the text.

θ (°)	0	30	60	90	120	150
$\Sigma\phi_D/\Sigma\phi_O$	1.15	1.08	1.11	1.17	1.26	1.30
$10^4 E_D$(max.)	32.60	32.00	4.80	0.02	0.04	0.44
$10^4 E_O$(max.)	0.69	4.25	0.19	0.10	0.33	3.70
J (cm^3·mol^{-2})	Porphyringlobin–porphyringlobin 3.63×10^9		Porphyringlobin–oxyhaemoglobin 8.16×10^8		Porphyringlobin–deoxyhaemoglobin 2.03×10^9	

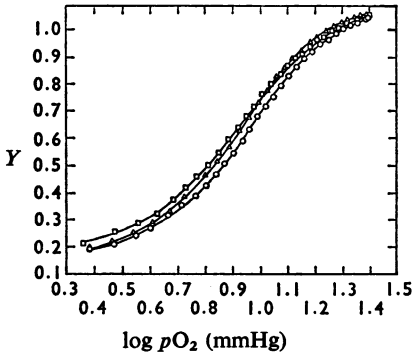


Fig. 4. *Fractional oxygen saturation at equilibrium* (Y) *as a function of oxygen partial pressure for haemoglobin* (△; 26.7 μM *dimers*), *a mixture of haemoglobin* (25.7 μM *dimers*) *with fast-moving porphyringlobin* (9.70 μM *dimers*) (□) *and a mixture of haemoglobin* (27.2 μM *dimers*) *and slow-moving porphyringlobin* (10.4 μM *dimers*) (○)

Potassium phosphate buffer (0.1 M; pH 7.0, 20°C) was used.

porphyringlobin has converted oxyhaemoglobin into the deoxy form. The thiol group reactivity of the hybrid shown in Fig. 2 indicates that the latter alternative is to be preferred. Ratio 5 derives from the ratios already discussed. Ratios 2, 4 and 6 are anomalous, apparently because transfer in the hybrid fast-moving porphyringlobin–oxyhaemoglobin is unduly favoured. Such a situation implies a tight structure with decreased Fe–Fe distances consistent with the observation that this hybrid alone has a dissociation constant that is smaller than those of the pure species contributing to its formation.

Oxygen equilibria of solutions of haemoglobin and porphyringlobin

Fig. 4 shows oxygen equilibrium curves for haemoglobin solutions in 0.1 M-potassium phosphate buffer, pH 7.0, both alone and in admixture with fast- and slow-moving porphyringlobin. The Hill exponent, n, for the haemoglobin equilibrium has the value 2.70, whereas p_{50} (oxygen partial pressure at half-saturation) = 8.19 mmHg. The addition of fast-moving porphyringlobin increases the affinity of the mixture for oxygen, $p_{50} = 7.85$ mmHg and decreases n to 2.43. A similar effect on the oxygen equilibrium curve of haemoglobin was observed after partial saturation with CO (Douglas *et al.*, 1912), partial oxidation (Darling & Roughton, 1942; Benesch *et al.*, 1965) or hybridization of methaemoglobin and deoxyhaemoglobin (Benesch *et al.*, 1965). According to Benesch *et al.* (1965) the effect arises because, in a hybrid of dimers with the liganded conformation (shared by methaemoglobin) and dimers with the deoxy conformation, the latter are forced into the liganded conformation of higher oxygen affinity. By analogy therefore we may conclude that fast-moving porphyringlobin affects the oxygen equilibrium curve by forming hybrids with deoxyhaemoglobin within which conformational change is transmitted. The direction of the change provides further evidence that fast-moving porphyringlobin has an oxy-like structure (Ainsworth & Treffry, 1974) and suggests that it imposes its structure on the deoxyhaemoglobin component of the hybrid. Referring, however, to a previous section, we presented evidence that the hybrid of deoxyhaemoglobin and fast-moving porphyringlobin reacts with 4-Py-S-S-4-Py at a rate that is more characteristic of deoxyhaemoglobin than it is of fast-moving porphyringlobin. We conclude therefore that the dimers of a hybrid affect one another, that conformational change is not unidirectional but that its apparent effects depend on the experimental system employed to observe it.

Fig. 4 also shows that adding slow-moving porphyringlobin to a haemoglobin solution decreases its affinity for oxygen, $p_{50} = 8.76$ mmHg and decreases the Hill exponent for the equilibrium, $n = 2.45$. We have already shown that slow-moving porphyringlobin decreases the thiol group reactivity of oxyhaemoglobin to that of itself when they are hybridized together. The decrease in oxygen affinity therefore arises because a proportion of the haemoglobin in solution is kept in the low-affinity deoxy conformation, even as oxygenation proceeds, by its presence in a hybrid with slow-moving porphyringlobin.

Table 3. *Experimental determination of* $\Sigma\phi_A/\Sigma\phi_B$

For details see the text.

Ratio ...	1	2	3	4	5	6
A	Fast-moving porphyringlobin–deoxyhaemoglobin	Fast-moving porphyringlobin–oxyhaemoglobin	Slow-moving porphyringlobin–deoxyhaemoglobin	Fast-moving porphyringlobin–deoxyhaemoglobin	Fast-moving porphyringlobin–deoxyhaemoglobin	Fast-moving porphyringlobin–oxyhaemoglobin
B	Slow-moving porphyringlobin–deoxyhaemoglobin	Slow-moving porphyringlobin–oxyhaemoglobin	Slow-moving porphyringlobin–oxyhaemoglobin	Fast-moving porphyringlobin–oxyhaemoglobin	Slow-moving porphyringlobin–oxyhaemoglobin	Slow-moving porphyringlobin–deoxyhaemoglobin
$\frac{F_A J_B}{F_B J_A}$	1.00	2.35	0.95	0.40	0.95	2.49

Fig. 5. *Hill plots from oxygen equilibrium data given in Fig.* 4

△, Haemoglobin. □, Calculated Hill plot for the hybrid deoxyhaemoglobin–fast-moving porphyringlobin, assuming that the hybrid concentration is 29.4% of that of the total haemoglobin dimers throughout. ○, Calculated Hill plot for the hybrid deoxyhaemoglobin–slow-moving porphyringlobin, assuming that the hybrid concentration is 18.6% of that of the total haemoglobin dimers throughout. *Y* is the fractional oxygen saturation at equilibrium.

It would be interesting to know whether the haemoglobin dimers within the hybrids retain any co-operativity. In principle, this problem may be answered by setting up an appropriate box equilibrium, but, unfortunately, the equilibrium constants involved in many of the steps are unknown. For this reason, we have made the drastic simplification of assuming that solutions of deoxyhaemoglobin and porphyringlobin may be treated as a mixture of the pure species and the hybrid whose proportions do not change as oxygenation commences and which react independently with oxygen. On this basis, the Hill exponent for the oxygen equilibrium of the two hybrids is 1.8 (Fig. 5). A second calculation, in which the proportion of hybrid present was interpolated linearly between the proportions that arise when the haemoglobin is either wholly deoxy- or oxyhaemoglobin, gave the Hill exponent for the oxygen equilibrium of the fast-moving porphyringlobin hybrid as 1.8 and that for the slow-moving porphyringlobin hybrid as 2.2. (The data used in this calculation were: fast-moving porphyringlobin–deoxyhaemoglobin = 29.4% of $[H_t]$; slow-moving porphyringlobin–deoxyhaemoglobin = 18.6% $[H_t]$; fast-moving porphyringlobin–oxyhaemoglobin = 28.0% $[H_t]$ and slow-moving porphyringlobin–oxyhaemoglobin = 32.0%

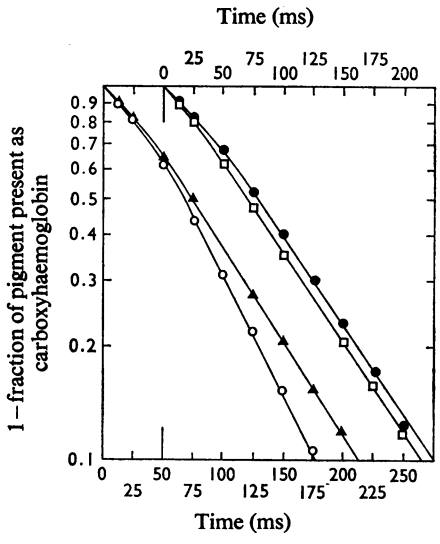


Fig. 6. *Time-course of the reaction of CO* (0.101 mM *before mixing*) *with deoxyhaemoglobin* (0.895 μM *dimers before mixing*) *alone* (●) *and in the presence of fast-moving porphyringlobin* (2.4 μM *dimers before mixing*) (□) (*in the solution* 76.5% *of the haemoglobin is present in the hybrid*) *and also with deoxyhaemoglobin* (1.775 μM *dimers before mixing*) *alone* (○) *and in the presence of slow-moving porphyringlobin* (1.625 μM *dimers before mixing*) (▲) (*in this solution* 29.2% *of the haemoglobin is present in the hybrid*)

Potassium phosphate buffer (0.1 M; pH 7.0, 25°C) was used. The reaction was monitored spectrophotometrically at 437.5nm.

$[H_t]$.) The nature of the assumptions required to derive the Hill exponents prevents too great a weight being placed on them: nevertheless, it is striking that n is so high (in one instance exceeding the theoretical maximum) and that its value appears to depend so little on the nature of the non-reactive dimer in the complex. Both observations, however, are consistent with Wyman's (1967) view that most of the co-operativity evident in the tetramer arises within its dimeric subunits.

Rate of CO binding

Fig. 6 shows that slow-moving porphyringlobin when added to a haemoglobin solution, decreases the rate at which CO is bound. Fig. 6 also gives data for another experiment (with a different sample of haemoglobin), which shows that added fast-moving porphyringlobin increases the initial rate with which haemoglobin binds CO: the final rate achieved, however, is no greater than that for haemoglobin alone. These effects are consistent with the equilibrium data: thus slow-moving porphyringlobin, present in a hybrid, permanently depresses the rate of binding by retaining its deoxy-like conformation. In contrast, fast-moving porphyringlobin hybrids have a greater rate because the haemoglobin dimer within the hybrid is forced into a conformation of higher affinity; this conformation, however, is achieved normally by the binding of haemoglobin tetramers so that the final rate observed is the same. The binding of CO in these experiments was monitored spectrophotometrically at 437.5nm, which is an isosbestic point for tetrameric and dimeric deoxyhaemoglobin (Kellett, 1971*b*). Note that there is no evidence in the reaction data for the porphyrin–haemoglobin mixture for the presence of a rapidly reacting component arising from the hybrid: this observation confirms that the haemoglobin component of the hybrids is subject to the constraints of the tetrameric form (Kellett, 1971*b*; Andersen *et al.*, 1971).

Discussion

The experiments described in the present and two preceding papers (Treffry & Ainsworth, 1974; Ainsworth & Treffry, 1974) have established that porphyringlobin exists in two forms which have some structural resemblances to oxy- and deoxyhaemoglobin, and that these pigments can react with haemoglobin derivatives to form hybrids in which the porphyringlobin dimers exercise a functional role consistent with their determined character. The significance of these findings lies in the fact that the porphyringlobin dimers of the hybrid do not bind ligands and that the concentration of the hybrid in mixtures of the parent compounds may be determined. The opportunity therefore exists to study the role of the dimer in tetrameric molecules whose structure may be compared with the deoxygenated and half-oxygenated states of haemoglobin.

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