Photolytic studies on the carbon monoxide complex of sulphaemoglobin

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The CO complex of sulphaemoglobin was found to be photolytically dissociable at pH 6.0 and pH 8.5. The recombination kinetics of the CO complex after flash photolysis show two phases, which differ in rate by approximately 20-fold. At both pH 6.0 and pH 8.5 the two phases show an identical linear dependence on CO concentration, with associated second-order rate constants of $6.0 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $1.2 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively. The percentage contribution of each of the two phases to the total absorbance change is independent of the CO concentration employed, but is a function of the protein concentration used. The percentage of the faster phase increases with protein dilution. These results taken together with computer simulations suggest the existence of appreciable amounts of monomeric species in solution at both pH 6.0 and pH 8.5 at low concentration of liganded protein. Estimates of the dissociation constants for the dimer $\rightleftharpoons$ monomer equilibrium yield values of $8 \mu\text{M}$ and $3.5 \mu\text{M}$ at pH 6.0 and pH 8.5 respectively. Stopped-flow delayed-flash-photolysis studies show that the liganded dimer decays to the liganded monomer with a half-life of approx. 0.5 s. Constant-illumination experiments allow an estimation of the half-life for the combination of deoxy monomers to deoxy dimers of 22 s. A model explaining the range of CO recombination kinetics seen for sulphaemoglobin does not require the existence of a photolytically produced high-activity form, even at high pH, in contrast with the case for normal haemoglobin.

Sulphaemoglobin is a characteristically green form of haemoglobin obtained by reaction of the protein with a source of thiol radicals (Nichol *et al.*, 1968), which leads to the introduction of a single sulphur atom into the porphyrin periphery with the consequent formation of this chlorin-like species (Morell *et al.*, 1967). The formation of sulphaemoglobin leads to the abolition of co-operativity within the molecule (Carrico *et al.*, 1978*b*; Brittain, 1981) and a decrease in O_2 -binding capacity to almost negligible proportions (Carrico *et al.*, 1978*b*). The protein, however, still retains the ability to combine with CO, although its affinity for this ligand is diminished relative to that of the native protein (Brittain, 1981). In an attempt to characterize this molecule further, flash-photolysis experiments have now been performed in order to probe the possibility of dissociation phenomena at low protein concentrations.

Some years ago it was realized that disaggregation has a significant role to play in the kinetic

behaviour of normal haemoglobin. A number of studies on the kinetics of the haemoglobin–CO complex (Gibson, 1959; Antonini *et al.*, 1967, 1968) showed that the behaviour of the protein is strongly pH-dependent. At high pH it appears that rapid photolysis leads to the transient formation of a form of the protein that is some 20 times more reactive than the normal form (Gibson, 1959). This reactive form decays to the normal species with a rate of approx. 200 s^{-1} . At low pH evidence has been presented suggesting that the kinetic behaviour observed in this region is best explained by the existence of a dimer $\rightleftharpoons$ monomer equilibrium of the ligated protein (Antonini *et al.*, 1968).

The present study is a thorough investigation of the characteristics of sulphaemoglobin subsequent to photolysis, and clearly indicates that no photolytically produced active form of the protein exists. The dominant kinetic features of this protein at both high and low pH are explicable in terms of the presence of a dimer $\rightleftharpoons$ monomer equilibrium that favours the dimer form in the deoxy species and the monomer form in the CO-ligated species, at low protein concentration.

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Experimental

Haemoglobin was prepared by the method of Drabkin (1946), and freed from catalase by passage through a column (5 cm $\times$ 50 cm) of Sephadex G-75 equilibrated with 0.1M-sodium phosphate buffer, pH 6.0. Sulphaemoglobin was prepared essentially as described by Nichol *et al.* (1968), except that a neutralized solution of ammonium sulphide was used as a source of sulphide instead of H₂S-saturated buffer. The progress of the reaction was monitored spectrophotometrically, and reaction was terminated after the spectral ratio A_{620}/A_{580} had reached at least 2.3. According to published data (Carrico *et al.*, 1978a), this would correspond to at least 90% sulphaemoglobin formation. The resulting sulphaemoglobin was passed down a column (2.5 cm $\times$ 25 cm) of Sephadex G-25, equilibrated with the buffer appropriate to the following experiments, to remove low-molecular-weight species used in the preparation. The concentration of sulphaemoglobin solutions was determined spectrophotometrically by using $A_{620} = 21.4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for the dithionite-reduced protein (Carrico *et al.*, 1978a). Static spectroscopy was performed in 1cm-path-length cells in a Cary 118c spectrophotometer.

Flash-photolysis experiments were performed with an Applied Photophysics flash-photolysis apparatus capable of delivering a 200J flash with a half-life of approx. 20 μs . A more detailed description of this experimental system has appeared previously (Edgerton *et al.*, 1980).

For stopped-flow delayed-flash-photolysis experiments the photolysis cell was filled directly from the mixing chamber of a Gibson-Milnes stopped-flow apparatus (Gibson & Milnes, 1964). The micro-switch of the stopping syringe was coupled to an electronic variable-delay unit that allowed delays of 10ms to 10s to be accurately interposed between the end of the flow period and the firing of the 200J photolytic flash. In continuous-illumination experiments the photolysis cell was illuminated at right-angles to the monitoring light-beam with a focused 200W lamp fitted with a heat filter. The length of illumination was varied by using a manually operated shutter.

All computing was performed with a PDP8/A minicomputer. Linear and non-linear least-squares fitting programs and enzyme kinetic simulation programs utilizing Runge-Kutta numerical integration procedures have been described previously (Barber *et al.*, 1978).

In all cases preliminary experiments were performed to ensure that total photolysis was occurring at the photolytic light-intensities employed. No light-induced irreversible changes occurred in the sulphaemoglobin solutions, as indicated by the absence of any overall spectral changes after repeated photolysis.

Results

When deoxysulphaemoglobin reacts with an excess of CO, the Soret band moves from 421nm to 418nm and increases in intensity by 10%. In the visible region the intense band at 620nm shifts to 613nm and increases in intensity by 25%. At pH 6.0 and pH 8.5 the process leading to these changes, as studied by flash photolysis, occurs in two distinct phases (Fig. 1). If the concentration of CO is varied, at constant protein concentration, the rates of the two phases are found to be linearly dependent on the CO concentration (Fig. 2). These plots yield second-order rate constants for the fast and the slow phases of $1.2 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $6 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively. It is noteworthy that these rates are independent of pH in the range studied, and the lower of the two rates corresponds to that obtained by stopped-flow methods (Brittain, 1981). The

Fig. 1. Typical reaction traces of the recombination of sulphaemoglobin with CO

Typical traces obtained when 25 μM -sulphaemoglobin recombines with 1 mM-CO at pH 6.0 (b) and at pH 8.5 (a) after a 50 μs 200J photolytic flash are shown. ● represents the best-fit curve to Scheme 1. See the text for details.

Fig. 2. Concentration-dependence of the rates of recombination of CO with sulphaemoglobin

The concentration-dependence of the slow (a) and the fast (b) phases seen on recombination of 25 μM-sulphaemoglobin with CO at 20°C are shown. Results were obtained at pH 6.0 (●) and at pH 8.5 (■).

Fig. 3. Ligand-concentration-dependence of the amounts of each phase in CO recombination with sulphaemoglobin

The ligand-concentration-dependence of the proportion of the total absorbance change associated with the rapid phase seen on recombination of CO with 25 μM-sulphaemoglobin at 20°C (●) is compared with that predicted by Scheme 1 (■). See the text for details.

percentage contribution of each of the two phases to the total spectral change is independent of the CO concentration employed either at pH 6.0 or at pH 8.5 (Fig. 3). The contribution of each phase to the over-

Fig. 4. Comparison of the CO combination difference spectrum obtained statically and the CO recombination difference spectrum obtained subsequent to flash photolysis

A comparison of the static (—) and kinetic difference spectra is shown, normalized to 55 μM-sulphaemoglobin in a 1 cm-path-length cell for the reaction of the protein with 1 mM-CO. The spectral contributions for the rapid (■) and the slow (●) kinetic phases are indicated.

all spectral change is independent of wavelength, and, as a consequence, the spectral distribution of the two kinetically resolved components is identical with the overall static difference spectrum when normalized to this spectrum at 610 nm (see Fig. 4).

If the protein concentration is varied and the CO concentration is held constant, then it is found that the proportion that the faster phase contributes to the overall spectral change increases substantially as the protein concentration is lowered from 100 μM to 1 μM (Fig. 5).

Discussion

In the case of human haemoglobin it has been proposed that at high pH photolysis of the haemoglobin-CO complex leads to the formation of an active species that can react with CO at an enhanced rate or else decay to the normally active form that is characterized by a CO recombination rate identical with that seen in stopped-flow experiments (Gibson,

Fig. 5. Effect of total protein concentration on the proportion of fast phase observed subsequent to flash photolysis. The fraction of fast phase (f) observed as a function of total sulphaemoglobin concentration (on a haem basis) is shown for pH 6.0 (a) and pH 8.5 (b). In both cases results were obtained with 1 mM-CO at 20°C.

Scheme 1. Possible CO recombination mechanism

1959). If this were the case for sulphaemoglobin, then the reactions seen in flash photolysis would be represented by a scheme such as that shown in Scheme 1, in which Hb^* and Hb represent the photolytically produced active form and the normal form of the protein.

To test if such a scheme was operative in the case of sulphaemoglobin, an attempt was made to fit the observed reaction profiles with those predicted by such a scheme. In order to do this, it was necessary to model the system by using a Runge-Kutta numerical integration computer program to predict the temporal changes of the various species after photolysis by solving the appropriate simultaneous differential equations:

$$\frac{d[\text{Hb}^*]}{dt} = -k_1[\text{CO}][\text{Hb}^*] - k_2[\text{Hb}^*]$$

$$\frac{d[\text{Hb}]}{dt} = k_2[\text{Hb}^*] - k_3[\text{CO}][\text{Hb}]$$

$$\frac{d[\text{HbCO}]}{dt} = k_1[\text{CO}][\text{Hb}^*] + k_3[\text{CO}][\text{Hb}]$$

At high pH and high protein concentration it was possible to obtain a good fit between predicted and observed reaction profiles by using the values for k_1 and k_3 obtained from CO-concentration-dependence studies and a value of 125 s^{-1} for k_2 obtained by iterative procedures (Fig. 1a).

When further tested, this scheme predicts an increase in the proportion of the rapid phase, due to k_1 , as the CO concentration employed is increased. This prediction clearly does not correlate with the observed data (Fig. 3). Further, a scheme such as Scheme 1 predicts that the proportion of each of the two reaction phases should be independent of total protein concentration. The experimental findings show a marked increase in the proportion of slow phase as the protein concentration is increased. In line with the proposed existence of a monomerization process in normal ligated haemoglobin (Antonini *et al.*, 1968), the protein-concentration-dependence observed in the present study was analysed according to a dimer = monomer equilibrium.

It can easily be shown that for a dimer = monomer system the fraction of monomer present (f) is directly related to the total monomer concentration ($[\text{Hb}]$) by:

$$\frac{1-f}{f^2} = 2K[\text{Hb}]$$

where K is the dissociation constant for the equilibrium (Antonini *et al.*, 1966). For the case of

sulphaemoglobin it was assumed that the faster phase is due to monomeric species, as is the case with normal haemoglobin, and that the molar absorbance change associated with each phase is the same (see Fig. 4). The appropriate plots (Fig. 5) show good straight lines, supporting the suggestion of a dimer = monomer equilibrium. The plots yield equilibrium constants of $3.5\mu\text{M}$ and $8\mu\text{M}$ for pH 6.0 and pH 8.5 respectively. It should be pointed out that, although these data indicate that a dimer = monomer equilibrium is present, it is not possible to determine from these data whether the monomer species represents the α - β 'monomer' in equilibrium with tetramer or else single-chain monomers in equilibrium with α - β -dimers.

It would thus appear that, for sulphaemoglobin, unlike normal haemoglobin, the characteristic form of the reaction profiles can be fully accounted for by the existence of a monomer = dimer equilibrium at both pH values. For this system it would also be necessary that the rates of monomerization be slow relative to the rates of ligand binding. Such a scheme would have the form shown in Scheme 2, in which Hb_D and Hb_M represent the dimer and monomer forms of sulphaemoglobin respectively. Further studies on this system were made with the use of different techniques. For example, if the dimer = monomer equilibria are slow and also, as is the case with normal haemoglobin, if the deoxy form is present largely in the dimeric form (Ip & Ackers, 1977), it is possible to determine the rate of monomerization of the ligated dimer, k_2 (Gibson & Antonini, 1967). If predominantly dimer deoxysulphaemoglobin, at low protein concentration, is made to react rapidly with

an excess of CO in the stopped-flow apparatus, then the initial product would be ligated dimer, which, according to the above data, should decay into the ligated monomer. Thus, if the rapidly formed material is photolysed, at various times after mixing, the proportion of the faster phase, which is due to monomeric forms, should increase with time. This is the case, as shown in Fig. 6. The data show that at both pH 6.0 and pH 8.5 the half-time for the decay of ligated dimers to monomers is approx. 0.5 s. This information, together with the equilibrium constant obtained from the protein-concentration-dependence experiments, allows an evaluation of the rate of dimerization of the ligated monomers (k_1).

Some information on the dimerization process involving the deoxy form of the protein may be obtained from constant-illumination experiments (Antonini *et al.*, 1968). In this case rapid photolysis of a low concentration of ligated protein yields the equilibrium values of the monomer and dimer present in solution, whereas if the protein is illuminated for an extended period, such that the ligated CO is 'held off', then the unligated protein re-equilibrates to favour the deoxy dimer form. As the length of illumination is extended, the concentration of dimer in the deoxy form increases, and on termination of the illuminating beam the proportion of fast-reacting material seen on CO recombination decreases. A plot of the percentage of fast-reacting material (Fig. 7) as a function of time of illumination thus allows an estimate to be made of the rate of dimerization of the deoxy monomer (k_3). For sulphaemoglobin, the rate of this process ($k_3 = 3.0 \times 10^{-8} \text{M}^{-1} \cdot \text{s}^{-1}$) is found to be signifi-

Fig. 6. Time-delay-dependence of the proportion of fast phase of CO recombination with sulphaemoglobin observed in stopped-flow flash-photolysis experiments

The proportion of fast phase observed when $2\mu\text{M}$ -sulphaemoglobin recombines with 0.5 mM -CO at 20°C after a 200J photolytic flash as a function of the delay between mixing and photolysis is shown at pH 6.0 (●) and pH 8.5 (■). The continuous line shows an exponential plot with $k = 1.5\text{ s}^{-1}$ for comparison.

cantly lower than that observed for the normal protein species ($k_3 = 10^{-6} \text{ M}^{-1} \cdot \text{s}^{-1}$; Antonini *et al.*, 1968).

The adequacy of Scheme 2 may be judged by a comparison of the observed and predicted effects of CO and protein concentration on the time course of CO recombination subsequent to flash photolysis. No value is available for k_4 , but, by analogy with the normal haemoglobin data, both it and k_3 may be assumed to be so small as to be ineffective under the conditions employed in the CO recombination kinetics. That this is indeed the case may be seen by the invariance of the percentage of fast phase as a

Scheme 2. Sulphaemoglobin-CO recombination mechanism

Fig. 7. Effect of constant illumination on the proportion of the fast phase of CO recombination with sulphaemoglobin

The Figure shows the proportion of the fast phase observed at pH 6.0 (●) and pH 8.5 (■) when $1 \mu\text{M}$ -sulphaemoglobin in the presence of $10 \mu\text{M}$ -CO is photolysed for various periods before recombination at 20°C . The continuous line in both cases is that expected for an exponential process with a half-life of 22 s.

function of CO concentration (Fig. 3), which indicates that no equilibration occurs between the unliganded forms of the protein during the time of a recombination experiment. With this simplification Scheme 2 has been used to predict the time courses of the recombination of CO with sulphaemoglobin under specific conditions. Fig. 8 shows a comparison

Fig. 8. Comparison of observed reaction traces with those predicted by Scheme 2

(a) shows the observed (—) and predicted (●) effect of CO concentration on the form of CO recombination curves obtained subsequent to flash photolysis at pH 8.5. The curves are for $25 \mu\text{M}$ -sulphaemoglobin with $1000 \mu\text{M}$ - (i), $350 \mu\text{M}$ - (ii) and $105 \mu\text{M}$ -CO (iii). (b) illustrates the effect of total sulphaemoglobin concentration observed (—) and predicted by Scheme 2 (●) on the form of CO recombination curves. The curves are for 1 mM -CO with $2 \mu\text{M}$ - (i), $10 \mu\text{M}$ - (ii) and $100 \mu\text{M}$ -sulphaemoglobin (iii) at pH 8.5. All curves are normalized to the same overall absorbance change.

Table 1. Comparison of some kinetic parameters for the CO recombination reaction of haemoglobin and sulphaemoglobin

	Haemoglobin pH 7.0	Sulphaemoglobin	
		pH 6.0	pH 8.5
(1) Parameters as related to Scheme 2			
$k_{\text{slow}} (\text{M}^{-1} \cdot \text{s}^{-1})$	$1.5 \times 10^{5*}$	6.5×10^3	6.5×10^3
$k_{\text{fast}} (\text{M}^{-1} \cdot \text{s}^{-1})$	$6 \times 10^{6*}$	1.2×10^5	1.2×10^5
(2) $K = k_1/k_2$			
$K (\mu\text{M})$	2*	8	3.5
$k_2 (\text{s}^{-1})$	0.5†	1.5	1.5
$k_3 (\text{M}^{-1} \cdot \text{s}^{-1})$	$10^{-6\dagger}$	2.4×10^{-8}	2.4×10^{-8}
$k_1 (\text{M}^{-1} \cdot \text{s}^{-1})$	$2.2 \times 10^{5\dagger}$	2×10^5	5×10^5

* From Edelstein *et al.* (1970).† Antonini *et al.* (1968).

of observed and predicted data. The match is quite good and serves to confirm the adequacy of Scheme 2 in accounting for the observed CO recombination time courses.

In summary, the characteristics of the CO recombination kinetics of sulphaemoglobin show a close resemblance to those seen for normal haemoglobin at low pH, as shown in Table 1. In both cases the kinetic complexity of the CO recombination process is explained by the existence of monomer=dimer equilibria at low protein concentration that favour the ligated monomer ($K \approx 1 \mu\text{M}$) and the deoxy dimer (Ip & Ackers, 1977). For sulphaemoglobin the increased tendency towards dissociation is no doubt due to the modification induced at the haem group, and is evident by the abolition of co-operativity between subunits, which is indicative of lowered inter-subunit interaction (Edelstein *et al.*, 1970). However, uncertainty remains as to the exact nature of the monomer species in both cases. For normal haemoglobin, for which there is a much larger body of information available, there is still dispute. Antonini *et al.* (1968) favour the interpretation of the monomer as single chains, whereas Edelstein *et al.* (1970) and Ip & Ackers (1977) favour the interpretation of the 'monomers' as $\alpha\beta$ -dimers and the 'dimers' as in fact $\alpha_2\beta_2$ -tetramers. At present insufficient data exist on the sulphaemoglobin system to identify specifically the nature of these equilibrium components. It should be pointed out, however, that this in no way affects the overall interpretation of the observations reported above.

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References

- Antonini, E., Bucci, E., Fronticelli, C., Chiencone, E., Wyman, J. & Rossi-Fanelli, A. (1966) *J. Mol. Biol.* **17**, 29–46
- Antonini, E., Chiencone, E. & Brunori, M. (1967) *J. Biol. Chem.* **242**, 4360–4366
- Antonini, E., Brunori, M. & Anderson, S. (1968) *J. Biol. Chem.* **243**, 1816–1822
- Barber, D., Parr, S. R. & Greenwood, C. (1978) *Biochem. J.* **175**, 239–249
- Brittain, T. (1981) *Biochim. Biophys. Acta* **673**, 253–258
- Carrico, R. J., Peisach, J. & Alben, J. O. (1978a) *J. Biol. Chem.* **253**, 2386–2391
- Carrico, R. J., Blumberg, W. E. & Peisach, J. (1978b) *J. Biol. Chem.* **253**, 7212–7215
- Drabkin, D. L. (1946) *J. Biol. Chem.* **164**, 703–723
- Edelstein, S. J., Rehmar, M. J., Olson, J. S. & Gibson, Q. H. (1970) *J. Biol. Chem.* **245**, 4372–4381
- Edgerton, M. E., Moore, T. A. & Greenwood, C. (1980) *Biochem. J.* **189**, 413–420
- Gibson, Q. H. (1959) *Biochem. J.* **71**, 293–303
- Gibson, Q. H. & Antonini, E. (1967) *J. Biol. Chem.* **242**, 4678–4681
- Gibson, Q. H. & Milnes, L. (1964) *Biochem. J.* **91**, 161–171
- Ip, S. H. C. & Ackers, G. K. (1977) *J. Biol. Chem.* **252**, 82–87
- Morell, D. B., Chang, Y. & Clezy, P. S. (1967) *Biochim. Biophys. Acta* **136**, 121–130
- Nichol, A. W., Hendry, I., Morell, D. B. & Clezy, P. S. (1968) *Biochim. Biophys. Acta* **156**, 97–108