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STUDIES ON LIGAND BINDING TO SULPHAEMOGLOBIN

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Summary

Equilibrium studies show that, at low protein concentrations, sulphaemoglobin, in the ferrous form, binds carbon monoxide at pH 6.0 in a non co-operative manner with a Hill coefficient of 1.15 and an affinity constant of $8 \cdot 10^{-7}$ M.

At both pH 6.0 and pH 9.0 the kinetics of CO binding show the presence of a simple mono-exponential process with a second-order rate constant of $8 \cdot 10^3$ $M^{-1} \cdot s^{-1}$. The rate of dissociation of CO from sulphaemoglobin is approx. 0.01 s^{-1}. The activation energy of the binding process is calculated as 40 kJ · mol^{-1}. A comparison is presented between the CO binding properties of sulphaemoglobin, myoglobin and haemoglobin and a mechanism whereby the CO binding parameters of sulphaemoglobin are modified is proposed.

Introduction

It has long been known that under the appropriate conditions haemoglobin can react with a source of sulphide to yield the bright green haemoprotein sulphaemoglobin [1,2]. In vivo sulphaemoglobin may be induced by administration of such drugs as phenacetin and sulphonamides [3]. Little was known of the mechanism of formation of sulphaemoproteins until Nicholls [4] showed that sulphmyoglobin could be prepared from purified myoglobin via the ferryl derivative. Nichol et al. [5] extended these earlier findings to show that almost quantitative conversion of oxyhaemoglobin to sulphaemoglobin could be achieved using a system containing ascorbate, H_2S and phenylhydroxylamine.

Sulphaemoglobin contains a modified chlorin-type haem in which one atom of sulphur has been introduced into the cyclic tetrapyrrole system [6].

Although plausible structures of the product haem have appeared [6] no direct characterisation has been carried out.

The binding of oxygen to sulphaemoglobin has been somewhat controversial in that all earlier workers claimed a complete lack of reactivity. Recently however one group has reported a study at low temperature, and high oxygen concentration, and have obtained a very low affinity constant for the binding of oxygen to the protein [7,8]. In comparison sulphaemoglobin is known to combine with carbon monoxide, but almost no quantitative measurements have appeared. In this paper we report the measurement of both equilibrium and kinetic parameters for the reaction of sulphaemoglobin with carbon monoxide. A comparison is made between the carbon monoxide binding to sulphaemoglobin, myoglobin and haemoglobin.

Materials and Methods

Phenylhydroxylamine was prepared by the zinc reduction of nitrobenzene [9], and was recrystalised from chloroform/petroleum ether (b.p. 60—80°C). The dry crystals were stored as small samples in stoppered containers, under an atmosphere of oxygen-free nitrogen, at —80°C, to prevent oxidation.

Carbon monoxide, oxygen-free nitrogen and hydrogen sulphide were supplied as compressed gasses by New Zealand Industrial Gasses, Auckland, New Zealand.

Purified haemoglobin and sulphaemoglobin were prepared essentially according to the methods of Nichol et al. [5]. The efficiency of conversion to the sulphaemoglobin from was monitored by measuring the ratio $A_{622}A_{582}$, of the deoxygenated protein using a value of 2.6 for 100% conversion [7]. All the samples used in these studies had a ratio of >2.3 and co-chromatographed, as a single species, with native haemoglobin both on Sephadex G-100 and carboxymethyl cellulose ion exchange. The experimental samples thus correspond to ⩾90% sulphaemoglobin, in the presence of less than 10% native haemoglobin, according to the criteria of Nichol et al. [5] and Carrico et al. [7]. Carbon monoxide titrations were performed by the anaerobic addition of small aliquots of standardised carbon monoxide equilibrated buffer to a known volume of protein solution in the presence of a slight excess of sodium dithionite. The end point of each titration was obtained by placing the protein sample used under an atmosphere of pure carbon monoxide.

Optical measurements were performed using an Aminco DW2a spectrophotometer. Kinetic experiments were performed using a computer-coupled stopped-flow system previously described [10]. All kinetic analyses were carried out as previously reported [11].

All experiments were performed at 19°C in 0.1 M sodium phosphate buffer, pH 6.0 unless stated otherwise.

Results

Equilibrium studies

When titrations with carbon monoxide were performed using protein solutions at concentrations of ⩾50 μM, linear plots of partial saturation versus car-

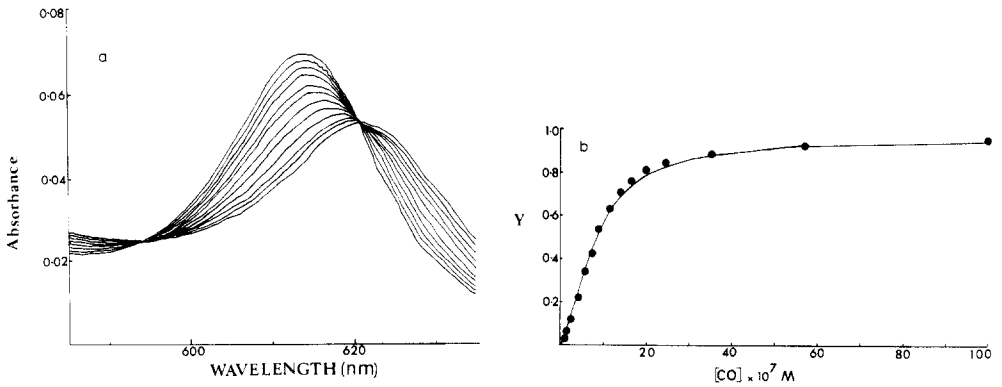

Fig. 1. A titration curve of 2.5 μM sulphaemoglobin with 10^{-4} M CO at pH 6.0 (a) and a plot of the fractional saturation Y (•) versus CO concentration with a non-linear least squares best fit of this data (———) (b).

bon monoxide concentration were obtained, which showed a stoichiometric relationship between haem and carbon monoxide concentration. At low protein concentrations (approx. 2 μM) hyperbolic binding curves were obtained (Fig. 1a and 1b). Either non-linear least squares computer fitting of the binding curve (Fig. 1b) or Hill plot analysis showed the presence of essentially non-cooperative binding and a high affinity constant (n = 1.15, $K_d = 8 \cdot 10^{-7}$ M).

In all cases good isosbestic points were obtained, showing that the carbon monoxide binding process involves only two spectrally active species.

Kinetic studies

In the pH range 6.0—9.0 ferrous sulphaemoglobin when rapidly mixed,

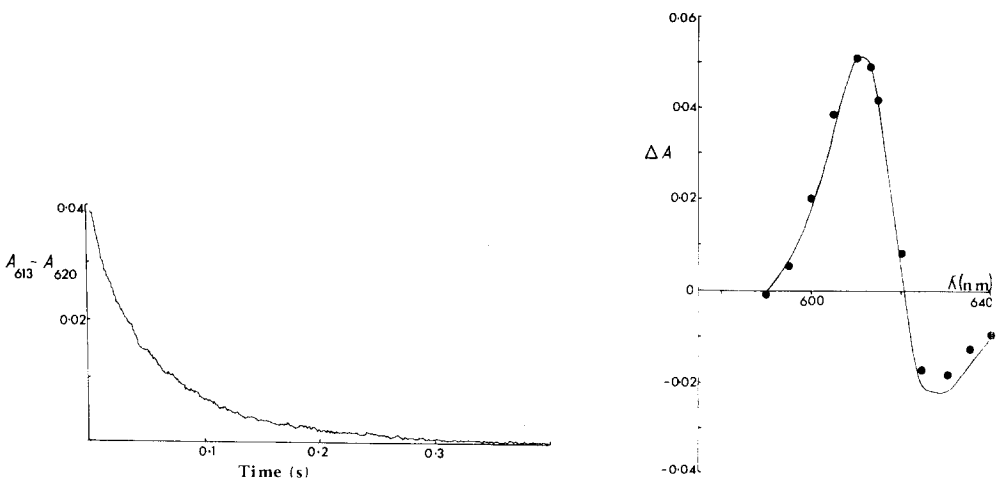

Fig. 2. A typical reaction curve obtained for the reaction of 4 μM sulphaemoglobin with an excess of CO (0.5 mM) at 19°C and pH 6.0.

Fig. 3. A comparison of the static (———) and kinetic (•) difference spectra between sulphaemoglobin and sulphaemoglobin + excess CO at a protein concentration of 5 μM.

anaerobically, with excess carbon monoxide showed a simple exponential reaction curve (Fig. 2) which gave semi-log plots which were linear for >97% of the reaction period.

A comparison of the difference spectra, obtained both kinetically and statistically, between the starting material and the final product (Fig. 3) showed them to be essentially identical; so proving that no spectrally active process was being overlooked in the dead-time of the stopped-flow apparatus.

At all pH values, at a fixed protein concentration, the reaction showed a linear dependence on carbon monoxide concentration, in the range of 30 μM to 0.5 mM. The slope of this linear plot yields a value for the second order rate constant for the reaction of $8 \cdot 10^3\ M^{-1} \cdot s^{-1}$. The very small positive intercept allows an estimate to be made of the first order rate constant, for the dissociation of carbon monoxide from the haems of sulphaemoglobin, of $\sim 0.01\ s^{-1}$.

The rate of reaction of sulphaemoglobin with carbon monoxide was found to be temperature dependent. An analysis of this dependence allowed the calculation of the activation energy of the binding process and yielded a value of $40\ kJ \cdot mol^{-1}$.

Discussion

Table I shows a comparison of the carbon monoxide binding parameters of sulphaemoglobin, myoglobin and haemoglobin. Clearly the binding of carbon monoxide to sulphaemoglobin is not drastically altered from that observed in haemoglobin, except for a lower binding rate and in particular the removal of the co-operative effect (indicated by the Hill number) normally observed in the reactions of haemoglobin. The later change in the characteristic of sulphaemoglobin could arise if the protein were irreversibly dissociated into its subunits by the process involved in its formation. However as sulphaemoglobin is eluted from a calibrated Sephadex G-100 column with exactly the same elution volume as native haemoglobin, under the conditions at which the above experiments were performed, another explanation for the loss of co-operativity must be found.

Some insight into this problem may be gained by a study of the previously reported oxygen binding studies [8]. Sulphaemoglobin has been found to bind oxygen with an affinity constant which is two to three orders of magnitude lower than that found for the native protein. This drastic reduction in affinity

TABLE I

A COMPARISON OF THE CO BINDING PROPERTIES OF SOME HAEMOPROTEINS

k_{on}, CO binding rate ($M^{-1} \cdot s^{-1}$); k_{off}, CO dissociation rate (s^{-1}); K_D, CO dissociation constant (M); n-Hill coefficient; $E_a^{\neq}$, activation energy ($kJ \cdot mol^{-1}$); a—f, Ref. 12—17.

	Sulphaemoglobin	Myoglobin	Haemoglobin
k_{on}	$8 \cdot 10^3$	$3 \cdot 10^5$,b	$3 \cdot 10^5$,f
k_{off}	0.01	0.017[b]	0.012[d]
K_D	$8 \cdot 10^{-7}$	$3 \cdot 10^{-8}$,a	$5 \cdot 10^{-8}$,c
n	1.15	1.0[6]	2.3[c]
$E_a^{\neq}$	40	44[b]	44[e]

has been accounted for by the suggestion that a delocalisation of π-electron density occurs away from the haem iron atom to the porphyrin periphery. This delocalisation leads to a weakening of the charge transfer between iron and oxygen which is responsible for the ligand binding [17—22]. The existence of this delocalisation was supported by the shift in the CO stretching frequency to 1961 cm^{-1} in sulphaemoglobin. Recent infrared studies have shown that the CO-stretching frequency in haemoproteins is in fact more sensitive to the relative position of the proximal and distal histidine residues [23]. Added to this a study of the binding kinetics of carbon monoxide to ferroporphyrins has indicated that the rates of binding are insensitive to π donor-acceptor interactions at the porphyrin periphery but are strongly affected by the hydrogen bonding patterns of the proximal histidine residues [24].

It would thus appear that both the carbon monoxide binding parameters and the previously reported oxygen binding studies can be best explained not by electron delocalisation into the porphyrin but by a shift of the histidine residues following the chemical modification of the haem. Any such shift of the liganded proximal histidine would certainly affect the affinity of the iron atom for carbon monoxide and oxygen by a trans-effect [25] mediated by the iron dz^2 orbital. Considering the intimate role envisaged for the proximal histidine residue in the co-operative functioning of haemoglobin [26], even a small movement of this residue would drastically alter the inter-subunit communication so necessary in the process of allostery. Although this suggestion explains the observed findings the proof must necessarily await more detailed information on the structure of the haem, and its surroundings, in sulphaemoglobin.

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