The Kinetics of Binding of Oxygen and Carbon Monoxide to *Gastrophilus* Haemoglobin

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The dimeric haemoglobin in the tracheal cells of the *Gastrophilus* larva was extracted and purified, and the spectral properties of its oxy- and carbon monoxide adducts are recorded. In dilute solutions the kinetic parameters of binding of oxygen and carbon monoxide were determined. In solutions between 0.1 and 50 μ M for oxygen k_{on} is $1 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ and k_{off} is 1 s^{-1} ; for carbon monoxide k_{on} is $6.5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ and k_{off} is 0.14 s^{-1} . These values are in agreement with previous equilibrium results on oxygen binding and carbon monoxide/oxygen partition. These results are discussed and compared with the known values for other monomeric protohaem proteins.

In contrast with the universal occurrence of haemoglobin in vertebrates, its presence in the invertebrates is phylogenetically spasmodic, and in insects, which form by far the largest group of invertebrates, haemoglobin is exceptionally rare. It occurs in a few Hemiptera and in the larvae of certain Diptera.

The object of this work is to study the haemoglobin of the larval stage of the dipteran genus *Gastrophilus*, which live as parasites in the stomach of the horse. The haemoglobin, first described by Keilin & Wang (1946), is present only in one stage during the development of the insect. Neither the pupa nor the mature insect possesses the pigment, which is present in special tracheal cells that are an integral part of the tracheo-spiracular system of the larva.

Four findings by Keilin & Wang (1946) were of special interest to us: (1) the haemoglobin is dimeric at high protein concentrations, with a molecular weight of 34000; (2) the O_2 dissociation curve shows no co-operativity between the two subunits of which it is composed; (3) the O_2 affinity, as measured by p_{50} values, shows a dramatic change on dilution of the protein, from values of 585 N/m² (4.9 mmHg) at 39°C and approx. 1 mm-haem to values less than 2.63 N/m² (0.02 mmHg) at the same temperature with 0.084 mm-haem; (4) the partition constant between CO and O_2 is 0.67 for this haemoglobin, in contrast with normal values of 120–500 for vertebrate haemoglobin.

These findings suggested that the haemoglobin was of exceptional interest and the kinetics of the binding of O_2 and CO to it are reported in this paper.

Materials

Gastrophilus haemoglobin was prepared as follows. Larvae of *Gastrophilus intestinalis* in the third instar

were harvested from the epithelial surface of the stomachs of freshly killed horses. They were washed free from adventitious material, and the tracheal system was dissected out by an incision below the spiracular plate. The complete system was removed and washed extensively with 0.9% NaCl, and the haemoglobin in the tracheal cells of 200–1000 larvae was leached out by osmosis in water overnight at 4°C. The red solution was made 70% saturated with $(\text{NH}_4)_2\text{SO}_4$, at 4°C, and the precipitate, which consisted of large amounts of glycogen, was removed by centrifugation at 15000g for 15 min. The saturation with $(\text{NH}_4)_2\text{SO}_4$ was increased to 82%, and the red haemoglobin was collected, dialysed and stored at 4°C in solution or as a slurry in saturated $(\text{NH}_4)_2\text{SO}_4$.

The material underwent rapid oxidation to methaemoglobin. To prepare oxyhaemoglobin, a concentrated solution of methaemoglobin (5 ml) was placed on a column (2 cm $\times$ 10 cm) of Sephadex G-25 equilibrated in 0.2 M-sodium phosphate buffer, pH 7.0, containing 0.1 mM-EDTA. The immediate addition of 10 mg of $\text{Na}_2\text{S}_2\text{O}_4$ caused reduction to deoxyhaemoglobin, which, separating from the dithionite on the column, took up O_2 from the buffer phase and emerged from the column as oxyhaemoglobin. Such a solution had a half-life, before reconversion into methaemoglobin, of 6 h at 20°C.

Buffer solution was prepared from stock solutions of 1 M- NaH_2PO_4 and 1 M- Na_2HPO_4 and was 0.2 M, pH 7.0. All water was double glass-distilled.

Reagents were of analytical quality where possible.

Methods

Spectra were measured with a Cary model 14 recording spectrophotometer with 1 cm-light-path cells in a thermostatically controlled cuvette holder.

Stopped-flow kinetic determinations were made

with a Gibson-Durrum instrument (Gibson & Milnes, 1964) with a 2cm observation tube. Band widths were 1–2nm and the dead-time of the instrument was 3–4ms.

Temperature-jump experiments were performed with the apparatus described by Eigen & De Maeyer (1963). The reaction was monitored at 435nm. A cell with a 1cm light-path was used. At temperatures of 20°C temperature jumps of 5°C were applied by using a discharge voltage of 30kV.

Flash-photolysis experiments used a specially constructed apparatus (Brunori *et al.*, 1972). The actinic beam was filtered with a Chance filter 5543 on the observation tube. Slits were 0.4mm with monochromatic light of 436nm. The path length of the experimental cuvette was 2cm on observation and 1cm on irradiation (100J, 100 μ s duration). The temperature was $\sim$ 25°C.

The kinetics of CO dissociation were measured by adding $K_3Fe(CN)_6$ (final concn. 1 mM) to a solution of CO-haemoglobin in a spectrophotometer cuvette and following the extinction change at 423 nm.

Sedimentation was performed in a Beckman Spinco model E analytical ultracentrifuge operated under standard conditions. Sedimentation coefficients were obtained from the movement of the maximum ordinate of the schlieren pattern or from the point

of inflexion of the densitometer trace of absorption films, and were corrected to water as solvent at 20°C.

For isoelectric focusing an LKB isoelectric-focusing electrophoretic apparatus with wide-range Ampholine carriers (pH 3–10) was used. Power (1 W) was dissipated at 4°C for 36h and the column was then eluted. The protein loading was 10mg.

Results and Discussion

Purity

The haemoglobin liberated from the tracheal cells of the *Gastrophilus* larvae, after purification, produced a single band (of methaemoglobin) with pI 6.27. This value is in good agreement with that reported by Keilin & Wang (1946). No heterogeneity was observed in any preparations, although the haemoglobin was derived from thousands of individual animals. In this the haemoglobin differs markedly from that derived from the larvae of *Chironomus* studied by Braunitzer (1970), where 12 distinct components were recorded.

In sedimentation studies with protein concentrations of 10mg/ml the maximum of the schlieren ordinate and the inflexion of the absorption profile corresponded exactly.

Fig. 1. Absolute absorption spectra of *Gastrophilus* deoxyhaemoglobin, oxyhaemoglobin and CO-haemoglobin

Molecular weight

The values for the sedimentation coefficients of *Gastrophilus* haemoglobin and methaemoglobin were determined in 0.2M-sodium phosphate buffer, pH7.0. All sedimentation data are corrected to water as solvent at 20°C. The values for methaemoglobin covered the range 2.0–10mg/ml and showed an unchanging sedimentation coefficient with a value of $2.92 \pm 0.115S$. This is consistent with the value reported by Adair *et al.* (1946).

The haem content of the preparation was measured as pyridine haemochromogen (Antonini & Brunori, 1971, p. 10). The value obtained was 3.90%. This corresponds to an equivalent weight of 17600, although the sedimentation data are consistent with a molecular weight of 35000. It is therefore concluded that the molecule is dimeric, as suggested by Keilin & Wang (1946).

Spectra

The absolute spectra of *Gastrophilus* haemoglobin and of its oxy- and CO adducts are shown in Fig. 1.

Fig. 2. Plot of $\log(\Delta E_0/\Delta E_t)$ versus time for the recombination of flash-dissociated CO-haemoglobin at pH7.0

The concentration of protein was $3.8\mu M$ and the concentration of CO was $25\mu M$. The slope of the curve is equal to $l'/2.303$, where l' is the pseudo-first-order constant in s^{-1} .

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The concentrations were expressed as millimolar in haem, as determined by pyridine haemochromogen measurements.

Kinetics of CO binding

Combination experiments. The kinetics of this process were measured by following the time-course of recombination of flash-dissociated CO-haemoglobin. The haemoglobin was $3.8\mu M$ and the CO present in solution was varied between 100 and $2.5\mu M$ in five separate experiments. The recombination process was homogeneous, and Fig. 2 shows a graph of $\log(\Delta E_0/\Delta E_t)$ against time (ms) for an experiment with $3.8\mu M$ -haemoglobin and $25\mu M$ -CO in 0.2M-sodium phosphate buffer, pH7.0. Fig. 3 shows a plot of the pseudo-first-order rate constant against ligand concentration. Under conditions where the ligand is in great excess the rate measured is essentially:

$$\begin{aligned} -\frac{d}{dt}[\text{haemoglobin}] &= \frac{d}{dt}[\text{CO-haemoglobin}] \\ &= l''[\text{haemoglobin}] \end{aligned}$$

Fig. 3. Reaction of *Gastrophilus* haemoglobin ($3.8\mu M$) with CO in 0.2M-sodium phosphate buffer, pH7.0

The pseudo-first-order rate constant is plotted against ligand concentration.

Fig. 4. Rapid-mixing experiments in which $15.6\mu\text{M}$ -protein is combined with $50\mu\text{M}$ -CO in 0.2M -sodium phosphate buffer, $\text{pH}7.0$

The results are plotted as $\log(\Delta E_0/\Delta E_t)$ versus time.

Fig. 5. Variation in pseudo-first-order rate constant with ligand concentration in rapid-mixing experiments with *Gastrophilus haemoglobin* and CO in 0.2M -sodium phosphate buffer, $\text{pH}7.0$

where $l'' = l' [\text{CO}]$ and l' is the observed pseudo-first-order rate constant. Thus Fig. 3 reveals a combination constant of deoxyhaemoglobin for CO of $6.3(\pm 0.2) \times 10^5 \text{M}^{-1} \cdot \text{s}^{-1}$ at 30°C .

In varying the protein concentration in the range between 75 and $4.7\mu\text{M}$ at a constant concentration of CO insignificant changes in the pseudo-first-order rate constant were observed. It appears that there is no change in the observed kinetic constants with haemoglobin concentration within this range.

These findings were confirmed by rapid-mixing experiments in which a solution of deoxyhaemoglobin was mixed with various concentrations of CO when $\text{Na}_2\text{S}_2\text{O}_4$ was present. Fig. 4 shows the change in $\log(\Delta E_0/\Delta E_t)$ with time for an experiment in which $15.6\mu\text{M}$ -protein was rapidly mixed with $50\mu\text{M}$ -CO. Fig. 5 shows the variation of pseudo-first-order rate constant with ligand concentration. The dependence of the rate on ligand concentration corresponded to a second-order process. The value obtained for the association rate constant was $5.6(\pm 0.2) \times 10^5 \text{M}^{-1} \cdot \text{s}^{-1}$ at 20°C . Again no change was observed in the rate constant when the concentration of the protein solution was varied between 50 and $15.6\mu\text{M}$.

The agreement between the two values obtained by flash and flow techniques, allowing for the difference in temperature in the two experimental conditions, supports the belief that the dissociation process is kinetically simple.

CO dissociation. The rate constant for this process was determined by mixing a solution of CO-haemoglobin ($12\mu\text{M}$) with $\text{K}_3\text{Fe}(\text{CN})_6$ (final concn. 1mM) at 22°C . The reaction was monitored at 423nm , and in triplicate experiments the half-time of conversion of the CO-haemoglobin into methaemoglobin was 5.2s . The dissociation rate-constant is therefore 0.13s^{-1} at 23°C .

Reaction with O_2

Temperature-jump experiments. A stock solution of oxyhaemoglobin was prepared as described in the Materials section and was $14.4\mu\text{M}$. A 1.5ml volume of this solution was mixed with 10ml of deoxygenated buffer and placed in the cell of a temperature-jump apparatus. By using the technique of relaxation spectroscopy, a single relaxation time was noted when the contents of the cell were subjected to a 5°C temperature-jump. In every case the approach to equilibrium showed only this one relaxation time, whose reciprocal was $340 \pm 25 \text{s}^{-1}$ with an O_2 concentration of $35\mu\text{M}$. Neglecting the dissociation rate constant (see below), this gave a value for the combination velocity constant of $0.97 \times 10^7 \text{M}^{-1} \cdot \text{s}^{-1}$ at 27°C . Because of technical difficulties these experiments were restricted to one protein concentration.

Dissociation velocity constant of O_2 from oxyhaemoglobin. The rate of dissociation of O_2 can be studied directly in experiments in which oxyhaemoglobin is rapidly mixed with a buffer solution containing $\text{Na}_2\text{S}_2\text{O}_4$. Two freshly prepared solutions of oxyhaemoglobin were used, and a series of protein con-

Fig. 6. Rapid-mixing experiments with 40 μM-oxyhaemoglobin and buffer containing Na₂S₂O₄ at pH 7.0. The progress of the reaction is plotted as log(ΔE₀/ΔE_t).

centrations was derived from these by serial dilution covering the concentration range from 350 to 1.4 μM. The reaction follows a simple first-order behaviour, as shown in Fig. 6, where the values of log(ΔE₀/ΔE_t) are plotted against time in an experiment in which 40 μM-oxyhaemoglobin was mixed rapidly with a solution of buffer containing Na₂S₂O₄. On varying the protein concentration over a range from 40 to 0.62 μM, the dissociation velocity constant for O₂ from oxyhaemoglobin was found to be 0.89 ± 0.23 s⁻¹ at 20°C and was independent of protein concentration across the range studied.

General Discussion

The results of the kinetic examination of the binding of O₂ and CO to *Gastrophilus* haemoglobin are summarized in Table 1, where relevant data of other simple protohaem proteins are assembled. A noteworthy feature of the kinetic constants of *Gastrophilus* haemoglobin is the low value for the O₂ dissociation rate constant, which is an order of magnitude lower than that observed for mammalian myoglobins and much lower than that observed for the molluscan myoglobin of *Aplysia* and for the lamprey haemoglobin. It is also clearly apparent that the association rate constant for O₂ is remarkably similar within the monomeric species reported, varying over only a twofold range. It is thus obvious that the affinity of a haemoprotein is controlled almost entirely by the magnitude of the dissociation rate

Table 1. Comparison of O₂ and CO binding constants for some single-chain haemoproteins

Protein	O ₂ binding			CO binding			Partition constant <i>M</i>	Reference
	<i>k</i> _{on} (M ⁻¹ .s ⁻¹)	<i>k</i> _{off} (s ⁻¹)	<i>K</i> (M ⁻¹)	<i>l</i> _{on} (M ⁻¹ .s ⁻¹)	<i>l</i> _{off} (s ⁻¹)	<i>L</i> (M ⁻¹)		
Horse myoglobin	1.4 × 10 ⁷	11	0.8 × 10 ⁶	3 × 10 ⁵	0.017	3.9 × 10 ⁷	39	Antonini & Brunori (1971, chapter 9)
Sperm-whale myoglobin	1.9 × 10 ⁷	10	1.9 × 10 ⁶	5 × 10 ⁵	—	—	—	
<i>Aplysia</i> myoglobin	1.5 × 10 ⁷	70	0.22 × 10 ⁶	4.9 × 10 ⁵	0.02	2.5 × 10 ⁷	106	Amiconi <i>et al.</i> (1972)
<i>Chironomus</i> haemoglobin	3.0 × 10 ⁸	218	1.4 × 10 ⁶	2.8 × 10 ⁷	0.095	3 × 10 ⁸	215	
Lamprey haemoglobin (monomer)	1.0 × 10 ⁷	80	0.125 × 10 ⁶	9 × 10 ⁵	0.09	1 × 10 ⁷	80	Andersen & Gibson (1971)
<i>Gastrophilus</i> haemoglobin	1 × 10 ⁷	1	10 × 10 ⁶	6.5 × 10 ⁵	0.14	4.5 × 10 ⁶	0.5	Present work

constant. It is this constant that is responsible for the high affinity of *Gastrophilus* haemoglobin for O_2 . The value of K reported in this paper ($1 \times 10^7 M^{-1}$) is in reasonable agreement with the value measured at low protein concentration by Keilin & Wang (1946), who quote a value of p_{50} of less than 2.63 N/m² (0.02 mmHg) at 39°C, which would be equivalent to a value of $K \leq 3 \times 10^7 M^{-1}$.

A further consistency is seen in the partition constant: $M = \frac{[HbCO] \cdot pO_2}{[HbO_2] \cdot pCO}$ which was shown by Keilin & Wang (1946) to be 0.67 for *Gastrophilus* haemoglobin. The value of K_{CO}/K_{O_2} obtained by compounding the kinetic constants in Table 1 is 0.5, which is consistent with the previous value. This value is at least two orders of magnitude lower than for any other respiratory haemoproteins.

It is therefore instructive to compare the CO-binding parameters of the various haemoproteins quoted. The combination velocity constants are about 20-fold slower for CO than for O_2 , the only exception to this rule being *Chironomus* haemoglobin, and the combination velocity constants show the same consistency as was seen in O_2 binding. The variation in affinity constant for CO is seen again mainly in the magnitude of the dissociation velocity constant, though there seems to be little correlation between the variation in this quantity and the corresponding dissociation rate constant for O_2 . The expression for this variability is then seen in the value of the partition constant, M .

These findings highlight the non-equivalence of these two gaseous ligands and exemplify the role played by the protein in modifying the micro-environment of the haem site.

The present study has concentrated on the kinetics of binding of O_2 and CO at low protein concentrations ranging from approx. 50 to 0.5 μM . Over this range no change in the rate constants with protein concentration was observed. The finding of Keilin & Wang (1946) that a 1 mM solution of *Gastrophilus* haemoglobin had a p_{50} value of 585 N/m² (4.9 mmHg) remains to be explored.

As to the physiological relevance of this pigment

in the larvae of *Gastrophilus*, much further work is needed. The animals live for long periods of time immersed in the fluid of the stomach, in what is assumed to be an anaerobic environment, at pH 7.4. The concentration of haemoglobin in the cells of *Gastrophilus* is approx. 1 mM. Thus the kinetics reported in the present paper for the dilute solution of the pigment probably have no direct physiological relevance, and efforts must be expended in deriving the values of the kinetic constants at concentrations approaching those found in the cells.

The present work indicates that the *Gastrophilus* haemoglobin is a single protein species. In this it offers advantages to protein chemists who may be troubled by the heterogeneity found, for instance, in *Chironomus* haemoglobin. The protein crystallizes easily to form bipyramidal prisms, and tryptic peptide 'maps' reveal 26 clearly demarcated spots. It might be expected therefore that X-ray crystallography and sequence determination would not be an arduous task with this protein, and, with the information then apparent, further insight into the means whereby the protein controls the reactivity of the haem centre would be forthcoming.

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