LIGAND BINDING KINETICS OF HEMOGLOBIN FROM THE EARTHWORM[1]

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Summary. Kinetic measurements of CO and O_2 binding by the high molecular weight (ca. 3,000,000) hemoglobin from Lumbricus terrestris reveal that: (1) the high co-operativity is associated with a much lower value for 1' ("on" constant for CO) than in mammalian hemoglobins and (2) the value of 1' for the subunits is at least 20 times that for the intact polymer. Co-operativity in O_2 binding is also manifested kinetically in the O_2 dissociation reaction in the absence and presence of CO as a replacement ligand.

The hemoglobin (or "erythrocruorin" (1)) from the earthworm (Lumbricus terrestris) shows a very high degree of co-operativity in the binding of oxygen, the n in Hill's equation (2) attaining a value of 5.4 (3). This protein, of molecular weight about 3,000,000 (4), is one of the largest of the known respiratory proteins, and is reported to have about 192 hemes (5) in the hemoglobin molecule. Studies of this protein by electron microscopy (6,7) reveal a structure consisting of 12 subunits, arranged with 2 regular hexagons face to face. This structure appears to dissociate into subunits (7) at pH 10. The subunits may contain 12-16 hemes, and in some cases appear to dissociate even further.

Materials. The worms were obtained locally from commercial sources. The hemoglobin was collected by cutting open the anterior portion of

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the ventral surface of the worm. The coelomic fluid was removed by blotting and the ventral vessel was cut. Hemoglobin was collected with a syringe as it flowed into the coelom. The hemoglobin was then centrifuged (1,000 x g) for ten minutes to remove particulate material. About 0.1 ml of hemoglobin, 1-2mM in heme, could be collected from each worm. For kinetic measurements, this hemoglobin was diluted into appropriate buffers. Potassium buffers 0.05M in phosphate were used for pH 6-8. The pH 10.3 buffer was 0.05M in borate. Dithionite solutions and argon and CO equilibrated buffers were made in standard ways (8). For determination of the dissociation velocity of O_2 in the stopped-flow apparatus, dithionite concentration was 0.1% by wt. in argon-saturated, oxygen-free buffers.

Approximate molecular weight determinations were carried out in columns of Sepharose 4B (Pharmacia) at pH 7, and in columns of Corning CPG-10 (700Å pore) column material at pH 10.3. In both cases, the columns measured 50cm x 0.9cm. The Sepharose 4B procedure was that of Marrink and Gruber (9). Thyroglobulin (Sigma, type 1), Apoferritin (Mann), Catalase (Worthington, crystalline), BSA (Sigma, crystalline), Chymotrypsinogen (Sigma, type 2), and Cytochrome c (Sigma, type 3) were used as marker proteins at pH 7. Catalase, BSA, and Myoglobin (Calbiochem, A grade) were used at pH 10.3 after deactivation of the column with BSA and hemoglobin. A model UA-2 ultraviolet analyzer (280 nm) and drop-detector (Instrumentation Specialties Co., Inc., Lincoln, Neb.) were used to monitor the columns. Blue Dextran 2000 (Pharmacia) was used in every run to fix the void volume. The concentration of hemoglobin upon elution from the column was 15 micromolar (heme-basis) at pH 7, and varied from 3-36 micromolar at pH 10.3.

Apparatus. Stopped-flow measurements were carried out in an apparatus designed by one of us (L.P.) to be described in detail elsewhere.

The optical path length is 2 cm., the cuvette volume is 63 microliters, 0.10-0.15 ml of each reagent are used per run, and the instrumental dead-time is about 1 msec. The temperature is monitored by a 1 mm diameter thermistor probe located 1 mm from the flow path in the rhodium and gold plated brass block, a single unit, which forms the valve-block, mixing chamber, and observation cuvette. Temperatures are read from a digital voltmeter to better than 0.01°. The light source is a 6V-18A GE CG101-AX ribbon filament lamp powered by a 1000w current-regulated power supply (10). The monochromator is a Jarrell-Ash 1/4-meter Ebert monochromator with 1 mm slits located between the light source and the reaction cuvette. The detection unit consists of a photomultiplier tube (EMI 9529B) with mu-metal shield, powered by a Kepco ABC-10, 0-1500V power supply. An operational amplifier follower circuit with adjustable time constant (1.4μsec-0.2sec) leads to a Tektronix R564B storage oscilloscope. The kinetic traces are photographed on 35 mm film and enlarged for data reduction.

Flash-photolysis measurements were made using commercial photographic electronic flash units: (a) Photogenic Speed Light, Model M1-28 (t1/2 of flash decay = 85 μsec) (b) Vivitar 160 (t1/2 =μ200 sec). The reaction vessels were various quartz cuvettes (path lengths 0.25, 1.00, 2.00, 10.00 mm) immersed in a water-filled cavity in a thermostated brass block. The brass block had 4 holes sealed with quartz windows at 90° intervals for flash-photolysis illumination perpendicular to the observing light path. The observation and detection instrumentation were as described above for the stopped-flow work, except for the addition of narrow-band interference filters at 3720Å and 4300Å just before the photomultiplier tube to reduce the optical disturbance from the flash.

Results and Discussion. The molecular weight of the hemoglobin as determined by gel-filtration is 2.5×10^6, in close agreement with

the value 2.73×10^6 reported by Svedberg and Eriksson (4). At pH 10.3, the molecular weight ranges between 120,000 and 200,000 depending on the initial concentration on the column. This is in excellent agreement with the electron microscopy of Levin (7) who reports seeing 1/12th subunits, and even smaller fragments (which would be less than 230,000 in molecular weight).

In the stopped-flow, one observes the reaction: $Hb + CO \xrightarrow{1'} HbCO$; in flash photolysis, the recombination reaction is studied: $HbCO \rightleftharpoons^{h\nu} Hb^{(*)} + CO$, where the photolysis product may be Hb* if the protein is cooperative in CO binding and the percentage photodissociation is small. The data for the reaction of this hemoglobin with CO are summarized in Table 1. The value of 1' for concentrated hemoglobin at pH 6-8 of $5\text{-}6 \times 10^4$ $M^{-1}sec^{-1}$ can be compared to $14 \times 10^4 M^{-1}sec^{-1}$ for human hemoglobin. This value is smaller than that reported in an earlier kinetic study by Gibson (11) of 22×10^4 $M^{-1}sec^{-1}$. We have occasionally also found this higher value, but only in instances where the protein was at very low heme concentration (3μM or less) or in 1% NaCl solutions. This value was not reproducible for us and may be associated with a transient dissociation to a half-molecule or hexamer

TABLE 1

COMBINATION RATE OF CO (1') WITH LUMBRICUS HEMOGLOBIN--20°C

Protein Conc.	Method	pH	$M^{-1}sec^{-1} \times 10^{-4}$	
15μM	flow	6	6.0	
2.5	flow	6	9.0	
9	flow	7	5.3	
3	flash	7.4	25	
440	flow	8	6.0	
102	flash	8	5.2	
90	flash	8	5.5	
30	flash	8	8.5	
4	flash	8	11	
3	flash	8	35	
3	flash	8	300	(Hb*)
60	flash	10.3	42	(Some Hb* present)
10	flash	10.3	120	
3	flash	10.3	200	

form, occasionally seen in electron microscopy (7). The data in Table 1 clearly show the dependence of the combination rate on protein concentration for concentrations near 30μM in heme. Dissociation of the 2.7 million molecular weight form in this region at all pH's studied could account for these results. Occasional differences between stopped-flow and flash-photolysis results in this dilute range are to be expected if the ligand-bound form of the protein is more dissociated than the reduced form, as is seen in the case of human hemoglobin (12). Owing to the greater sensitivity of the pH 10.3 form of the protein to dithionite, most of the kinetic studies have been made in O_2-free, dithionite-free buffers by flash-photolysis. There is a striking pH effect on the combination rate in going from pH 8 to pH 10.3. Only at the latter pH (and 2μM in heme) did Levin (7) observe extensive dissociation of the protein. From our gel-filtration work at pH 10.3 and 3μM in heme, it appears that the protein has dissociated into subunits which are of average mol. wt. corresponding to 1/24th the intact hemoglobin. The kinetic data show that at concentrations below 10μM at pH 10.3, co-operativity is essentially lost.

In Table 1, Hb* refers to quickly-reacting hemoglobin seen on partial breakdown of the HbCO in flash-photolysis, and which presumably has the ligand-bound conformation (13). It is of interest to note that the Hb* form of this protein, (seen in nearly all co-operative hemoglobins) as well as the Hb, or slowly-reacting form, seen at higher percentage breakdown of HbCO, can both be detected at pH 7. This means that the conformational change Hb* to Hb is more rapid than the ligand-binding reactions even in this large molecule. As might also be expected for a hemoglobin more highly co-operative than human hemoglobin, the Hb* form is seen only at much smaller fractional breakdown, making an accurate determination of 1' for Hb* even more difficult. We are able to observe but a single rate for 1' as a function of percentage breakdown for the 10μM pH 10.3 form of the protein. This rate, however,

TABLE 2

RATE OF OXYGEN DISSOCIATION--20°C

Protein Conc.	pH	k sec^{-1}	k* sec^{-1} (+CO)
30μM	7	45	10.6
10	7	33	20
3	7	33	23
30	10.3	15	7.0
10	10.3	8.8	7.0
3	10.3	7.3	6.9

is close to the Hb* pH 8 rate. This result may be compared to that found in human hemoglobin where the Hb* rate is very similar to that for the isolated non-co-operative subunits (14).

Another kinetic manifestation of co-operativity is seen in the oxygen dissociation reaction, the results of which are in Table 2. Gibson and Roughton (15) have shown that in a co-operative hemoglobin, subject to very general restrictions, the rates for the reaction of HbO_2 with dithionite in the presence and absence of CO should be different, for when CO is present, one is at all times observing the loss of O_2 from a fully-liganded (Hb*) form of hemoglobin. When CO is absent from the dithionite solution, one observes an average rate for the loss of O_2 from successive intermediates in the deoxygenation process. Our kinetic results for the pH 7 form at 30μM in heme, and the pH 10.3 form at 10μM in heme are in accord with value of $n > 4$, and approximately 1, respectively.

In Lumbricus hemoglobin, the high co-operativity is reflected in: (1) a larger ratio of 1' for Hb* to Hb than for human hemoglobin (60 vs. 30) (2) a larger ratio for the dissociation of O_2 in the absence and in the presence of CO than for human hemoglobin (>4 vs. 2.4), and (3) the appearance of Hb* at much lower fractional breakdown than for human hemoblobin. The high co-operativity apparently arises from interactions among the subunits of molecular wt. 120,000-200,000 which when isolated at pH 10.3 retain little, if any, co-operativity. Preliminary work indi-

cates that these subunits can largely re-assemble to form a co-operative hemoglobin when the pH is lowered to 7.

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