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Kinetics of Ligand Binding in the Hemoglobin of *Lumbricus terrestris*†

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ABSTRACT: Sodium dodecyl sulfate gel electrophoresis and determinations of the per cent Fe in the hemoglobin of *Lumbricus terrestris* indicate 1 heme/17,000. This finding suggests that the double-hexagonal hemoglobin structure revealed by electron microscopy consists of 12 subunits, each containing 16 hemes. Gel filtration molecular weight studies on the hemoglobin of *Lumbricus* show a decrease in molecular weight from 2.5×10^6 at pH 7 to 0.25×10^6 (and lower) at pH 10.3. The reactions studied, CO combination and oxygen dissociation, are sensitive to changes in pH and protein concentration. Kinetic measurements strongly suggest that there is little if any cooperativity in ligand binding in the isolated 16-heme subunits. The high cooperativity in ligand binding shown by the hemoglobin from *Lumbricus* must arise from interactions among the 16-heme subunits.

A kinetic study of ligand binding in annelid hemoglobins provides an excellent opportunity to correlate structure and function in a complex protein. The intact hemoglobin from *Lumbricus terrestris*, the earthworm, has been shown (Levin, 1963; Roche, 1965) to consist of 12 subunits, arranged in two superimposed regular hexagons, 265 Å in length (measured between opposite vertices within a hexagon), 160 Å in width, and 160 Å in thickness. The intact duodecamer of mol wt 3.2×10^6 (Rossi-Fanelli *et al.*, 1970) contains approximately 192 hemes, and is one of the largest of the known respiratory proteins. The duodecamer is known to dissociate first into hexamers, splitting perpendicular to the plane of the hexagons, and then into the one-twelfth subunits (Levin, 1963). Levin further reported that at pH 10.2 the dissociation into subunits

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appeared essentially complete, and that many of the one-twelfth subunits had even dissociated further. Oxygen binding by the intact hemoglobin from *Lumbricus* is highly cooperative, with $n = 5.4$ (Cosgrove and Schwartz, 1965) in Hill's equation (Hill, 1910). The first ligand kinetic studies on *Lumbricus* hemoglobin were carried out by Salomon (1941) who measured the rate of oxygen dissociation at pH 8 and 23°. Later, Gibson (1955) reported rates for oxygen dissociation at pH 6.7 and 9.2 and CO combination at pH 6.3 and 9.2, all measurements at 20°. We have previously reported (Boelts and Parkhurst, 1971) preliminary kinetic studies on this hemoglobin, and report here detailed results for oxygen dissociation and CO combination as a function of pH and protein concentration, as well as molecular weight studies on the hemoglobin and its subunits.

Experimental Procedure

Materials. The worms were obtained locally from commercial sources. An incision was made in the anterior ventral surface of the worm from the clitellum to the heart area and the coelomic fluid removed by blotting. The ventral vessel was cut and the hemoglobin collected with a 1-ml syringe. About 0.1 ml of hemoglobin, 1–2 mM in heme, could be collected from each worm. The pooled hemoglobin from several worms was then centrifuged (1000*g*) for 10 min to remove particulate material. All kinetic measurements were made on freshly collected hemoglobin. For the work reported here, blood from 600 worms was required. The concentrated hemoglobin was diluted into appropriate buffers for the kinetic measurements. Potassium buffers, 0.05 M in phosphate, were used for pH 6–8. At pH 9 and 10.3, the buffer was 0.05 M in borate. All chemicals were reagent grade and used without further purification. Solutions were equilibrated with argon and CO as described previously (Parkhurst *et al.*, 1970). For determination of the dissociation velocity of oxygen in the stopped-flow apparatus, dithionite concentration was 0.1% by weight in argon-saturated, oxygen-free buffers.

Methods. MOLECULAR WEIGHT determinations on the annelid hemoglobin were carried out in columns (50 × 0.9 cm) of Sepharose 4B (Pharmacia) at pH 7 and 9 according to the procedure of Marrink and Gruber (1969), and in Corning CPG-10 (700-Å pore) column material at pH 10.3. In both cases volumes were measured by counting drops. The columns were monitored by a Model UA-2 ultraviolet analyzer (280 nm) and drop detector (Instrumentation Specialities Co., Inc., Lincoln, Neb.). Blue Dextran 2000 (Pharmacia) was used in every run to establish the void volume. Marker proteins at pH 7 were thyroglobulin (Sigma, type 1), apoferritin (Mann), catalase (Worthington, crystalline), bovine serum albumin (Sigma, crystalline), chymotrypsinogen (Sigma, type 2), and cytochrome *c* (Sigma, type 3). At pH 10.3, catalase, bovine serum albumin, and myoglobin (Calbiochem, A grade) were used after the column had been deactivated with the albumin and several samples of annelid hemoglobin had been run. The flow rate was 6 ml/hr for the Sepharose column and 10 ml/hr for the Corning CPG column, all runs at 9°. Qualitative molecular weight determinations at pH 7 and 10.3 were made in a Spinco Model L preparative ultracentrifuge at 60,000*g*.

SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS was carried out by modifications of the procedure of Weber and Osborn (1969). Except for myoglobin and the annelid hemoglobin, the proteins were dissolved in 1% sodium dodecyl sulfate and 0.002 M iodoacetamide and incubated at a concentration of 2–10 mg/ml for 1 hr at 45°. Myoglobin and the annelid hemoglobin were incubated in 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol. After incubation at 2 mg/ml, the *Lumbricus* hemoglobin was concentrated fivefold by pervaporation. In some instances, the hemoglobin preparations were boiled for 5 min before pervaporation. In four runs, dithiothreitol (1 mg/ml) was used in addition to the mercaptoethanol. The *Lumbricus* hemoglobin contained a small percentage of non-heme protein with an approximate molecular weight of 300,000. To remove this contaminant, hemoglobin used for sodium dodecyl sulfate electrophoresis was purified by electrophoresis on a 5% acrylamide gel at 200 V for 45 min. The top portion of the gel containing the pure hemoglobin was cut out and the protein eluted into distilled water by soaking for several hours. The sodium dodecyl sulfate gels were 10% in acrylamide and 0.27% in *N,N'*-methelenebisacrylamide, and were cast in tubes 5 mm in diameter, 12 cm in length. They were polymerized by addition of 0.015 ml of *N,N,N',N'*-tetramethelenediamine and 0.5 ml of a saturated riboflavine solution. The gel buffer was 0.01 M in sodium phosphate (pH 7.0) and contained 0.1% sodium dodecyl sulfate. Stock solutions contained 0.04 ml of 10% Bromophenol Blue, 0.06 ml of glycerol, and 0.21 ml of the 0.5–1% protein solution. For the application of the sample, the split gel technique (Dunker and Rueckert, 1969) was used in which 0.02 ml of the solution containing the marker proteins was applied to one side of the gel, and 0.02 ml of the *Lumbricus* sample was applied to the other side of the same gel. The electrophoresis was carried out at 90 V for 2.5–3 hr. The gels were removed from the tubes and the proteins were fixed and stained by letting stand overnight in a methanol–water–acetic acid solution containing 2.5% Amido Schwarz in 50% methanol–9.2% glacial acetic acid. The gels were then removed from the staining solution, rinsed with distilled water, and placed in destaining solution (5% methanol–7.5% glacial acetic acid in water) for a minimum of 1 hr. The gels were then destained in 20 min electrophoretically in the destaining solution. The mobilities of the proteins were determined from the ratios of protein to Bromophenol Blue migration distances. A plot of the log of the molecular weight *vs.* protein mobility was then constructed, and from this plot the molecular weights of the subunits in *Lumbricus* were determined. In order to determine the relative intensities of the stained bands, the bands were cut out and eluted electrophoretically using 1% sodium dodecyl sulfate buffer. The absorbances of the eluted Amido Schwarz fractions were then determined at 620 nm from absorption spectra measured on a Cary 14 recording spectrophotometer.

CELLULOSE ACETATE ELECTROPHORESIS was carried out on globin from the annelid hemoglobin using Millipore electrophoresis cells (XE2100015) and strips (ESWM0310R). The strips were presoaked for at least 1 hr in 0.05 M pH 5.9 citrate buffer containing 0.5% 2-mercaptoethanol either with or without 6 M urea. Electrophoresis was carried out for 20–30 min at 125 V in a barbitol buffer (pH 8.45), 0.075 ionic strength, containing 0.5% 2-mercaptoethanol, with or without urea (6 M). The strips were stained with Amido Schwarz.

IRON CONTENT. In order to measure the per cent Fe in the protein, the protein concentration was determined by the Biuret method (Layne, 1957) on globin prepared from purified hemoglobin. The annelid hemoglobin was purified by ultracentrifugation at 100,000*g* for 1 hr. Globin was prepared by a modification of the method of Rossi-Fanelli *et al.* (1958), using an acetone solution containing 10 ml of concentrated HCl and 25 ml of glacial acetic acid per 500 ml of acetone. The heme splitting was carried out at 0°. The globin suspension

was centrifuged (500*g*, 10 min, 4°) and the globin was dissolved in 10% NaOH and the protein content was determined by the Biuret method using human globin as a standard. In order to correct for the presence of residual heme at 540 nm, an absorption measurement was made using another cuvet which contained globin at the same concentration. The iron content was determined from the absorbance at 558 nm of the pyridine hemochromogen (Falk, 1964) prepared from an aliquot of the hemoglobin used for the globin preparation.

KINETIC STUDIES. The stopped-flow and flash-photolysis devices have been described previously (Boelts and Parkhurst, 1971). During the later stages of the study, these kinetic devices were interfaced to a Supernova computer (Data General Corp.) following a design of R. J. De Sa (personal communication, 1971), and thereby provided us with on-line data reduction and kinetic analysis. In a typical run, 200 data points are collected and averaged in 20 groups of 10 points each. The 10 points within each group are averaged by fitting to Tchebycheff polynomials through degree 2 to produce from the original 200 points a final set of 20 smoothed points. The computer then computes first- or second-order rate constants between successive points as well as successive overall rate constants computed with respect to the initial smoothed data point and displays the numerical data on a storage oscilloscope within 1 sec after the completion of the reaction. Hard copy output is obtained on a KSR 35 teletype and by microfilming the oscilloscope display.

Results

Molecular Weight Studies. The apparent molecular weight of the annelid hemoglobin was 2.5×10^6 for runs in which the hemoglobin concentration was 15 μM (heme basis) upon elution from the column. Over the final 90% of the column length, the protein concentration decreases fourfold to that measured upon elution. The same peak position was observed for elution concentrations ranging from 7.6 to 24 μM at pH 7. When the elution concentration was 3.4 μM and lower, the peak position corresponded to 1.25×10^6. At a concentration of 1 μM in heme upon elution, a shoulder appeared at 650–750 $\times 10^3$, and a small peak at 110,000 was observed. At pH 9, and 15 μM upon elution, only 2% of the protein showed a peak at 2.5×10^6, for the main peak had shifted to 500,000 and showed considerable tailing. The apparent molecular weight at pH 10.3 varied from 68,000 to 230,000 as the hemoglobin concentration upon elution varied from 3 to 10 μM, respectively.

Seventy-five sodium dodecyl sulfate acrylamide electrophoresis experiments for the determination of the subunit molecular weights clearly showed two bands at 16,000 and 19,000, with an abundance ratio of 2–3:1, corresponding to an average globin weight of *ca.* 17,000. Occasionally, bands were observed corresponding to molecular weights in the 11,000–13,000 molecular weight range accompanied by a decrease in the amounts of the 19,000 and particularly the 16,000 weight band. Similar dissociation to fragments of this size in sodium dodecyl sulfate gels has recently been observed in other annelid hemoglobins (Waxman, 1971; Swaney and Klotz, 1971). The hemochromogen and biuret determinations for Fe and protein give a value of 17,000 ± 1000 for the average molecular weight of the protein per heme. Both HbCO[1] and Hb were centrifuged for 2.5 hr 60,000*g*, at pH 10.3. No pellet was observed for HbCO (10 μM, heme basis), whereas 40% of the Hb, 10 μM, formed a pellet, and at 30 μM, 50% sedimented. Thyroglobulin (mol wt 669,000) and catalase (mol wt 232,000) were studied under similar conditions. Catalase did not form a pellet, whereas 70% of the thyroglobulin sedimented. Under the same conditions at pH 7, more than 99% of the 10 μM HbCO formed a pellet. A sample of HbCO, 10 μM in heme, was incubated 45 min at pH 10.3, and then brought to pH 7, and centrifuged for 2.5 hr, 60,000*g*. Approximately 45% of this material formed a pellet in the ultracentrifuge.

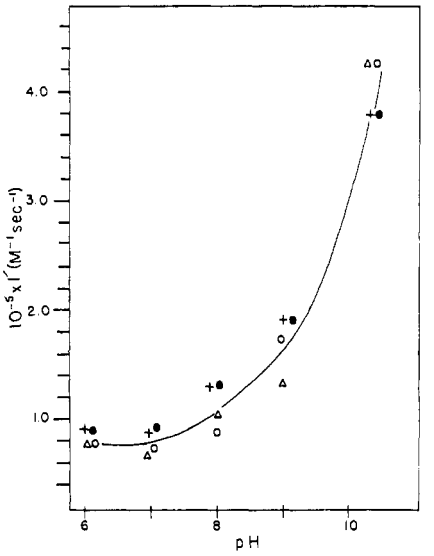

FIGURE 1: Rate constants (l') for the combination of carbon monoxide with *Lumbricus* hemoglobin as functions of protein concentration and pH as measured by stopped flow, 20°. Hemoglobin concentrations before mixing: (△) 60 μM, (○) 30 μM, (+) 10 μM, and (●) 3 μM.

Cellulose Acetate Electrophoresis. Electrophoresis on cellulose acetate strips of the globin from *Lumbricus* hemoglobin in 6 M urea gave four bands which migrated at pH 8.45 toward the anode. The four bands could be observed in the absence of urea, but the highest resolution was obtained when urea was present in the presoak, the running buffer, and in the globin solution. The four bands appeared to be present in approximately equal amounts.

Kinetic Studies. The various reactions which were studied in general showed homogeneous kinetic behavior and the rate constants reported in the Figures 1–4 are for the best fitting overall rate constant. Heterogeneity was observed only in the CO "on" reactions for concentrations 10 μM in heme and lower. In these instances, the reactions were often biphasic, with the slower phase representing between 80 and 93% of the total reaction. For these reactions, we report the rate constant for the slow phase.

Figure 1 depicts the results from stopped-flow experiments for the determination of the CO combination rate constant, l', for the reaction

$$\text{Hb} + \text{CO} \xrightarrow{l'} \text{HbCO} \qquad (1)$$

(The rate of the reverse reaction is negligible.) The protein concentration during the reactions varied from 60 to 3 μM (heme basis). Each point represents the average of at least 5 and in some cases, 20 determinations.

[1] Abbreviations used are: Hb, deoxyhemoglobin, HbCO, carboxyhemoglobin; HbO_2, oxyhemoglobin.

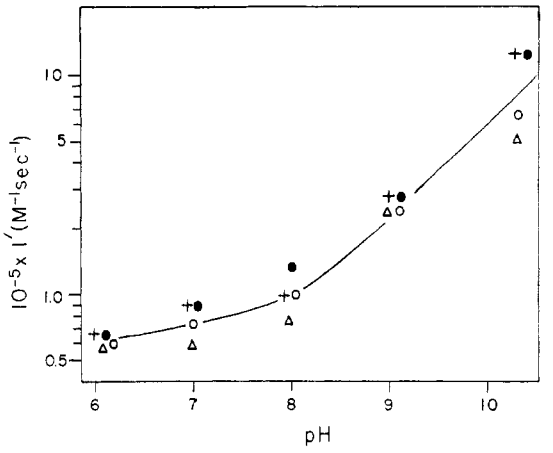

FIGURE 2: Rate constants (l') for the combination of carbon monoxide with *Lumbricus* hemoglobin as functions of protein concentration and pH as measured by flash photolysis, 20°. Hemoglobin concentrations are (Δ) 100 μM, (○) 30 μM, (+) 10 μM, and (●) 3 μM.

In Figure 2 are shown the results of flash-photolysis determinations of the CO recombination rate constant plotted on a logarithmic scale. In flash photolysis the HbCO is subjected to an intense burst of light ($h\nu$), and one observes the recombination of the CO to the heme site:

$$HbCO \underset{l'}{\overset{h\nu}{\rightleftharpoons}} Hb + CO \qquad (2)$$

The photolysis light was sufficiently intense that photodissociation of the HbCO was essentially complete. Thus, the reaction of Hb, and not Hb*, was observed. In Figures 3 and 4 are shown the results of studies on the rate of oxygen dissociation as a function of pH and protein concentration. The oxygen dissociation rates were determined from stopped-flow experiments in which the oxyhemoglobin solutions were mixed rapidly with the appropriate dithionite-containing buffers.

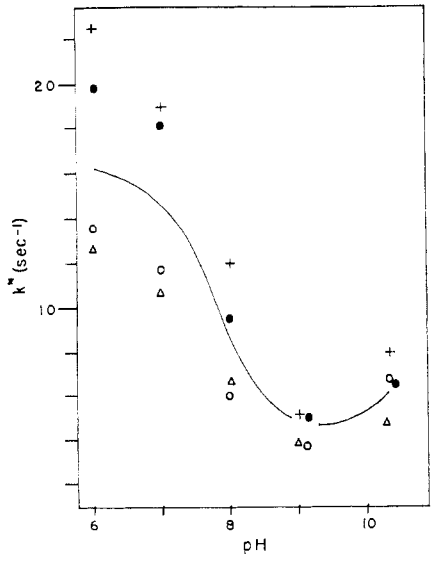

FIGURE 3: Rate constants (k^*) for the dissociation of oxygen from the ligand-bound conformation of *Lumbricus* hemoglobin as functions of protein concentration and pH, 20°, stopped-flow determinations. Hemoglobin concentrations (before mixing) are (Δ) 60 μM, (○) 30 μM, (+) 10 μM, and (●) 3 μM.

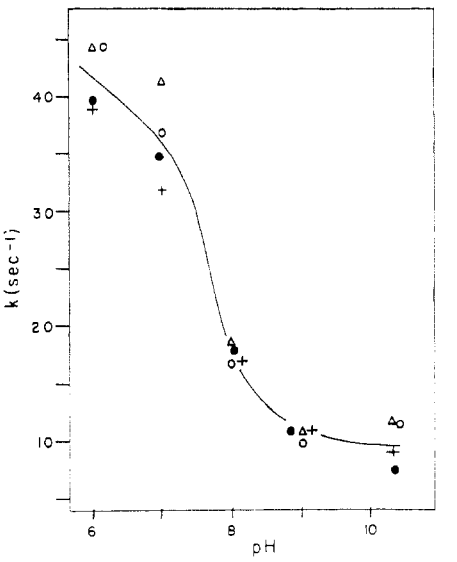

FIGURE 4: Rate constants (k) for the dissociation of oxygen from *Lumbricus* hemoglobin as functions of protein concentration and pH, 20°, stopped-flow determinations. Hemoglobin concentrations (before mixing) are (Δ) 60 μM, (○) 30 μM, (+) 10 μM, and (●) 3 μM.

For the experiments shown in Figure 3, the dithionite solutions were also saturated with CO. In these experiments, the rate constant for CO binding for the ligand-bound conformation (Hb*) is sufficiently large that one measures the rate with which the first oxygen molecule dissociates from fully liganded hemoglobin, since at all times the hemoglobin remains in the fully liganded state:

$$Hb(O_2)_n \longrightarrow HbCO(O_2)_{n-1} \longrightarrow HbCO_2(O_2)_{n-2} \cdots \longrightarrow Hb(CO)_n \qquad (3)$$

In Figure 4, CO is absent from the dithionite solution, and one measures an average of the rates for dissociation of successive oxygen molecules from the hemoglobin:

$$Hb(O_2)_n \longrightarrow Hb(O_2)_{n-1} \cdots \longrightarrow Hb \qquad (4)$$

pH-Jump Experiments. Experiments were carried out in which l' and k^* were measured within a few milliseconds of a sudden pH change for proteins 1–30 μM in heme. In some experiments, the protein, at pH 7.0 in 0.001 M potassium phosphate, was mixed in the stopped-flow with buffer at pH 10.75, 0.05 M in borate containing dithionite and CO. The final pH was 10.3. In these experiments, the rate measured (l' or k^*) was that characteristic of the protein at pH 10.3 within 10 msec of the pH change. (Ten milliseconds was the minimum time required to obtain a reliable value for the reaction rate constant.) In another set of experiments, the pH was suddenly dropped from pH 10.3 to 7 by mixing the protein at pH 10.3 in 0.001 M borate buffer with 0.05 M phosphate (pH 7.0) buffer, containing CO and dithionite. In these experiments, the rates measured were the same as for those measured at pH 7 for the protein (k^*) or biphasic (l'), with 65–75% of the protein reacting with a rate similar to that of protein equilibrated at pH 7. In a final set of experiments, protein was incubated at pH 10.3 for 15–20 min, and both k and k^* were measured following a rapid drop in pH for protein 3–10 μM in heme. In these experiments, k^* was 22 sec^{-1} and k was 25–27 sec^{-1} for both concentrations of protein. In these latter experiments we

hoped to obtain values for k and k^* for the one-twelfth subunit at pH 7, and thus arrive at a lower limit for the Hill parameter "n" for this subunit at the physiological pH.

Discussion

The molecular weight determinations by gel filtration are in agreement with light-scattering (Rossi-Fanelli *et al.*, 1970) and electron microscopy studies (Levin, 1963; Roche, 1965) showing that the species at pH 7 and 7.6 μM in heme is predominantly the intact duodecamer. At pH 10.3, our results at 10 μM suggest a one-twelfth subunit, and at 3 μM, further dissociation is evident. These findings are in accord with the electron microscopy of Levin at pH 10.2 for protein initially 2 μM in heme. At pH 9, 15 μM in heme, the protein appears to exist primarily in dissociated forms, one-fourth to one-sixth of the intact duodecamer. The qualitative ultracentrifuge runs show that the deoxyhemoglobin is much less dissociated at pH 10.3 than is the ligand-bound form. The reassociation upon lowering the pH from 10.3 to pH 7 after 45-min incubation at pH 10.3 is not complete, since only 45% could be obtained as a pellet at 60,000g, conditions under which 99% of the native hemoglobin will form such a pellet. The per cent Fe determinations show that the average weight per heme is *ca.* 17,000. This finding and the sodium dodecyl sulfate electrophoresis results imply that if the intact duodecamer has a molecular weight of 3.2×10^6, then there are approximately 192 hemes in the molecule, and that if the one-twelfth subunits are identical, they consist of hexadecamers. It is interesting that a hexadecameric hemoglobin was found in another annelid (*Glycera dibranchiata*) as the polymeric form, and was found to be noncooperative in oxygen binding (Seamonds, 1969). The species of mol wt 68,000 observed in gel filtration at pH 10.3 and 3 μM in heme suggests that dissociation has progressed to a tetrameric species. A species of this size was also observed in sodium dodecyl sulfate electrophoresis when mercaptoethanol was omitted. If such tetramers are all identical, then the cellulose acetate electrophoresis in urea suggests that each tetramer consists of four different chains, one with molecular weight *ca.* 19,000, and three with molecular weights of approximately 16,000. Such a model with a hexadecamer as the one-twelfth subunit is not in accord with results reported by Rossi-Fanelli *et al.* (1970). In that work, the Fe:protein ratio was obtained from a gravimetric measurement on dried hemoglobin, using human hemoglobin as a reference. A difference in residual hydration for the two proteins could account for their results. Our hemochromogen and biuret determinations suggest that perhaps three of the chains have very nearly the same number of amino acid residues as human hemoglobin, and that one has perhaps 10% more residues. In other respects, such as the low isoelectric point (pH 5.1 for *Lumbricus* hemoglobin), the geometry and molecular weight of the intact pH 7 form, the ligand equilibria and kinetics, the *Lumbricus* hemoglobin shows large differences from human hemoglobin.

Measurements of oxygen equilibrium on *Lumbricus* hemoglobin at pH 7.4 and 240 μM in heme, conditions under which the hemoglobin is largely in the form of the intact duodecamer, have yielded a value of $n = 5.3$ (Cosgrove and Schwartz, 1965). We were unable to study the oxygen equilibrium of the pH 10.3 form directly, owing to progressive oxidation of the hemoglobin during the course of the equilibrium measurements. Gibson and Roughton (1955) have shown, however, how measurements of the rate of oxygen dissociation can give one a lower bound for the Hill constant "n." In terms of our experiments, the relationship is: $k/k^* \leq n$. If the subunit dissociation rate in the annelid oxyhemoglobin were similar to that observed in a flow-flash experiment for human hemoglobin, $t_{1/2}$ *ca.* 1 sec (Gibson and Antonini, 1967), then the protein would be in that state of aggregation characteristic of its concentration *before* mixing in the stopped flow for the various oxygen dissociation studies, and it is the before-mixing concentration which should be compared to gel filtration results. Our results show that at pH 10.3, k/k^* ranges from 2.4 for 60 μM protein (concentration *before* mixing) to 1.1 for 3 μM hemoglobin. At pH 7, k/k^* ranges from 4 to 2, for hemoglobin 60 and 3 μM, respectively. The decrease in "heme–heme" interaction, or cooperativity, is thus correlated with a dissociation of the molecule into hexadecamers and smaller units. In the pH and concentration regions where the hexadecamer is found, the oxygen dissociation kinetics give only slight evidence for cooperativity in oxygen binding. It is difficult to make precise kinetic and molecular weight correlations, however, since at each pH there are undoubtedly species of varying molecular weights. At pH 10.3 and 10 μM in heme, however, the predominant species must be the hexadecamer (one-twelfth subunit). In order to study this species at pH 7, the physiological pH for the earthworm, we measured both k and k^* for hemoglobin 10 and 3 μM in heme (before mixing) within about 10 msec after the pH had been rapidly lowered to pH 7 in a stopped-flow apparatus. (If the subunit combination rate is similar to that for human α and β chains, *ca.* 1×10^5 M^{-1} sec^{-1} (Antonini *et al.*, 1966), the half-time for dimerization would be about 1 sec.) These measurements showed that the ratio k/k^* at pH 7 for the hexadecamer is 1.2, suggesting very little cooperativity in this subunit. These studies also showed that there is a pH dependence shown by the various reactions apart from the molecular weight changes induced by the pH variations. Cooperativity thus apparently arises primarily from interactions among the hexadecamer subunits. Within experimental error, there is no kinetic evidence for cooperativity in the oxygen-dissociation reactions for the 68,000 molecular weight form. Thus, in a sense, the hexadecamers in *Lumbricus* hemoglobin are analogous to the α and β chains in human hemoglobin in that the isolated structural units are not cooperative in ligand binding but the cooperativity in the intact hemoglobin undoubtedly involves conformational changes in these subunits. The rapid pH change experiments show that the protein can rapidly adjust to a pH increase to give ligand binding kinetics nearly identical with those observed for the same protein equilibrated at the final pH for several minutes. Differences are found for experiments in which the pH is rapidly lowered, however, indicating that complete reassociation does not occur.

As in human hemoglobin, another kinetic manifestation of cooperativity is the low value for l', the "on" constant for CO binding, in the unliganded, intact duodecamer at pH 7. With increasing pH and dilution, this rate constant increases until at pH 10.3, the rate is comparable to that for the Hb* form of the *Lumbricus* hemoglobin. This is very similar to the situation that obtains for human hemoglobin, in which the isolated chains have ligand-binding rates similar to that of Hb* (Geraci *et al.*, 1969). In the *Lumbricus* hemoglobin, the high cooperativity is associated with a value of l' which is about one-third that measured for human hemoglobin (Gibson, 1964). In Figure 1 are shown results from a stopped-flow determination of l' in which the initial species is the unliganded hemoglobin. In Figure 2 are flash-photolysis results, in which the protein is present in solution in the ligand-bound

form except for the few milliseconds between the photolytic flash and the completion of the reaction. It can be seen that at pH 6 and 7 and at high protein concentrations, the two methods yield similar values for l'. At pH 10.3, however, the flash-photolysis values are all higher than the flow results. This is in accord with our ultracentrifuge results which show that the deoxy form of the protein is more highly aggregated at pH 10.3 than is the ligand-bound form. This preferential dissociation of the ligand-bound form has been observed in other hemoglobins (Andersen *et al.*, 1971; Andersen, 1971).

A further kinetic manifestation of cooperativity in hemoglobins is the difference in l' for the unliganded (Hb) form of the protein and the Hb* ligand-bound conformation, seen on partial photodissociation of HbCO. In *Lumbricus* hemoglobin, Hb* is seen at a much lower fractional photodissociation than in human hemoglobin, implying that the Hb → Hb* transition occurs after a larger fraction of the hemoglobin has bound ligand than in human hemoglobin. A detailed account of these studies will be reported elsewhere (L. Parkhurst and K. Wiechelman, manuscript in preparation).

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Kinetics of Carbon Monoxide and Oxygen Binding for Eight Electrophoretic Components of Sperm-Whale Myoglobin†

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ABSTRACT: Sperm-whale myoglobin has been fractionated by isoelectric focusing in Sephadex gels, and oxygen and CO ligand association and dissociation kinetics were measured by stopped-flow and flash photolysis for the eight most abundant fractions. Except for the association rate for CO binding, there appeared to be no significant differences in rates among the various bands for a given reaction. Rates for oxygen dissociation and association determined by replacement reactions were in good agreement with rates determined with dithionite and by flash photolysis, respectively. The rate for CO dissociation determined by NO replacement was homogeneous. Heterogeneous kinetics were observed for the dissociation reaction when $Fe(CN)_6^{3-}$ was used. An evaluation of M, the O_2–CO partition constant, from the kinetic data was in excellent agreement with a direct equilibrium determination.

The electrophoretic heterogeneity of crystalline sperm-whale myoglobin has been known for at least 6 years. As many as five electrophoretic components have been observed on polyacrylamide gel electrophoresis (Hardman *et al.*, 1966) and cellulose acetate (Parkhurst,[1] 1968), while Hapner *et al.* (1968) isolated four components from CM-cellulose columns. When isolated components of many proteins, such as the hemoglobins of trout (Binotti *et al.*, 1971), eel (Yoshioka

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