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Kinetic Light Scattering Studies on the Dissociation of Hemoglobin from *Lumbricus terrestris*†

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ABSTRACT: The kinetics of the pH-induced dissociation of the 3×10^6 mol wt hemoglobin from *Lumbricus terrestris* (the earthworm) have been studied in a light-scattering stopped-flow apparatus. The ligand dependent dissociation data were fit well by a simple sequential model. The data for CO and oxyhemoglobin are consistent with $Hb_{12} \rightarrow 2Hb_6 \rightarrow 12Hb$. Methemoglobin at pH 7 appears to be hexameric and the dissociation is consistent with the model: $Hb_6 \rightarrow 6Hb$. In a sequential decay scheme for which light-scattering changes are monitored, the relative amounts of rapid and slow phase are determined by the rate constants as well as the molecular weights of intermediate species. Assignment of the hexameric intermediate is supported by an investigation of the sensitivity of the theoretical kinetic curves to the molecular weights of the intermediates. This assignment is further supported by the following: (1) the same model will fit the data for oxy- and CO-hemoglobin at all three temperatures (a 24–29-fold variation in rate constants), (2) evidence from electron microscopy shows hexameric forms, and (3) methemoglobin is apparently stable as a hexamer at pH 7. When CO replaces O_2 as the ligand, the dissociation rate *increases* by a factor of four. The met dissociation rate is about 20 times faster than the initial oxyhemoglobin dissociation rate, but perhaps more relevant for comparing dissociation of the hexamer, the met rate was respectively 100 times and 500 times faster than that for the assumed hexameric forms of CO- and oxy-hemoglobin. The activation energies for the dodecamer to hexamer dissociation and for the dissociation of the hexamer to smaller forms were about 30 kcal/mol for oxy-, CO-, and methemoglobin.

The hemoglobin from the earthworm (*Lumbricus terrestris*), one of the largest of the known respiratory proteins, has a molecular weight of about 3×10^6 (Rossi-Fanelli et al., 1970) and is reported to contain 192 hemes (Wiechelman and Parkhurst, 1972). It has been shown to be highly cooperative in binding oxygen, with a Hill number of 5.4 (Cosgrove and Schwartz, 1965). Electron microscopy studies of this protein reveal a structure consisting of 12 subunits, arranged with two regular hexagons face to face (Levin, 1963, Roche, 1965). The molecule has dimensions of 265 Å in length (measured between opposite vertices within a hexagon), 160 Å in width, and 160 Å in thickness (Levin, 1963; Roche, 1965). This oxyhemoglobin structure dissociates at pH 10.2 into the one-twelfth subunits (Levin, 1963) containing 12–16 hemes (Wiechelman and Parkhurst, 1972; Chiancone et al., 1972), and in some cases further dissociation occurs (Levin, 1963; Roche, 1965).

Ligand kinetic studies of this protein were first carried out by Salomon (1941) who measured the rate of oxygen dissociation at pH 8 and 23°. Later Gibson (1955) reported rates for CO combination at pH 6.3 and 9.2 and oxygen dissociation at pH 6.7 and 9.2, all at 20°. Wiechelman and Parkhurst (1972) have studied oxygen dissociation and CO combination as a function of pH and protein concentration and also reported that the deoxyhemoglobin was much less dissociated at pH 10.3 than was the ligand-bound form.

We wish to report here an extensive investigation of the kinetics of the pH-induced dissociation for various ligand forms of the protein.

Experimental Section

Materials

The worms were obtained locally from commercial sources and the hemoglobin was collected as described by Boelts and Parkhurst (1971). The hemoglobin was sedimented by centrifugation in a Spinco Model L ultracentrifuge at 85000*g* for 2.25 hr at 4°C. The hemoglobin pellet was dissolved in 0.001 *M* potassium phosphate buffer (pH 7). Dissociation was induced in the light-scattering, stopped-flow apparatus by flowing equal volumes of the protein in low ionic strength (1 m*M*) pH 7 potassium phosphate buffer against pH 10.7, 0.05 *M* borate buffer ("jump buffer") so that the final pH was 10.3. The initial hemoglobin concentration for dissociation measurements was 120 μM in heme (0.2%). There were no significant changes in

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kinetic properties from 3 to 40 hr following preparation of the hemoglobin. Results reported here are for measurements within this time span. Except for time in the stopped-flow while the experiments were being run, the solutions were maintained at 4°. The CO- and methemoglobin solutions were prepared in standard ways (Steinmeier and Parkhurst, 1975). At least two preparations of hemoglobin were used for each ligand and each temperature. A minimum of four runs were averaged for each ligand and temperature.

Qualitative ultracentrifuge runs were performed on freshly prepared HbCO[1] which was then incubated at pH 10.3 in CO saturated borate buffer for 10–15 min. The pH was lowered to 7 and the samples were centrifuged at 85000*g* as described above.

Methods

Kinetic studies were carried out in a specially constructed light-scattering stopped-flow interfaced (LaGow and Parkhurst, 1972) to a Super Nova mini-computer (Data General Corp.). The photomultiplier (EMI 9601B) was mounted above the quartz cuvette at right angles to the incident light beam (150-W xenon arc source, Jarrell-Ash ¼ meter Ebert monochromator, 547 nm, all quartz and mirror optics). The stopped-flow cuvette had entrance and exit apertures 2 mm^2 and an emission aperture 2 mm × 2 cm. Measured from the center of the cuvette, the photomultiplier half acceptance angle could be varied up to a maximum of 50°. The reactions were followed to 75–90% of completion.

Data Analysis. The data were fit according to three sequential models. The first model (I) is a general mechanism that does not distinguish the details of the hexamer dissociation process: $Hb_{12} \rightarrow 2Hb_6 \rightarrow 12Hb$. The general form of the equation is

$$A \xrightarrow{k_1} 2B \xrightarrow{k_2} 12C$$

Since the largest dimension of the molecule is less than 1/20th of the incident light wavelength, internal interference can be neglected and the scattered light voltage with background subtracted, V_t, is given by:[2,3]

$$V_t = a\Sigma m_i C_i \qquad (1)$$

where "a" is a constant, m_i is the molecular weight of the ith species, and C_i is the concentration in g/ml. If n_i is the number of moles/ml (1) can then be written as

$$V_t = a\Sigma m_i^2 n_i \qquad (2)$$

V_t/a' is then $(12)^2A_t + (6)^2B_t + C_t$, a' is a constant and A_t, B_t, and C_t are the molar concentrations of A, B, and C at time t. The scaled voltage change can be expressed as

$$R_t = (1/a')(V_t - V_\infty) \qquad (3)$$

Using eq 3 and the integrated expressions for A_t, B_t, and C_t

$$R_t/60 = 2.2A_0e_1 + [k_1A_0/k_{12}]e_{21} \qquad (4)$$

where $k_{ij} = k_i - k_j$ and $e_{ij} = \exp(-k_it) - \exp(-k_jt)$, $e_i = \exp(-k_it)$. In fitting R_t to the data A_0 was treated as a variable representing a scale factor times the initial concentration of A. For a general two-step dissociation (model Ia):

$$Hb_{12} \xrightarrow{k_1} MHb_{12/M} \xrightarrow{k_2} 12Hb$$

$$R_t = A_0(132e_1 - (12 - 144/M)k_1e_{21}/k_{21}) \qquad (5)$$

The partial derivatives of R_t with respect to A_0, k_1, and k_2 were used for a three parameter weighted least-squares fit using the Fletcher–Powell algorithm (Fletcher and Powell, 1963) sum of squares minimization program on the Super Nova computer with initial parameter values varied over a grid extending from 0.5 to 5 times the final parameter values.

Two other models were examined which differed from model I in that the dissociation of the hexamer was considered in detail. We felt it necessary to consider these models in order to limit the hypothetical reverse reactions to bimolecularity. Model II: $Hb_6 \rightarrow 2Hb_3 \rightarrow 2Hb_2 + 2Hb$; $2Hb_2 \rightarrow 4Hb$. Model III: $Hb_6 \rightarrow Hb_4 + Hb_2$; $Hb_2 \rightarrow 2Hb$, $Hb_4 \rightarrow 2Hb_2 \rightarrow 4Hb$. The differential equations of models II and III are readily solved by Laplace transform methods with inversions derived from the residues of the Bromwich (Fourier–Mellin) integral (Bromwich, 1916) and/or the Faltung theorem (Wagner, 1940). Data were fit to models II and III using the Fletcher–Powell sum of squares minimization program with numerical derivatives on an IBM 360-65 computer.

These models, with the exception of Ia, assumed that the dodecamer split first to a hexamer. The rationale for this will be discussed later. For the slow phase of the reaction, attributed to the hexamer dissociation, we have investigated the only other two models consistent with the restriction that each cleavage be dichotomous and along a single plane through the hexamer or a fragment of the hexamer.

Results

The dissociation rates for CO and O_2-bound forms of the hemoglobin at various temperatures are given in Table I. The data were fit with the sequential models as described above. The rate constant k_1 varied no more than 4% for all three models. The second phase of the reaction could be fit well by a single exponential decay. Models II and III both assumed that three constrained first-order processes contributed to this phase. Since these rate constants were highly correlated and the improvement in the fit over that obtained from model I as judged both by the sum of squares and the number of residual runs (Swed and Eisenhart, 1943) was negligible, we report rate constants only for model I, with the understanding that k_2 represents an average rate constant for at least three steps. For both HbO_2 and HbCO at all temperatures, however, the rate constant for initial decay of the hexamer (models II and III) varied no more than 20% from that reported for k_2 (model I).

[1] Abbreviations used are: HbCO, carbon monoxide-hemoglobin; HbO_2, oxyhemoglobin.

[2] The time-dependent turbidity correction (attenuation of incident light) at these protein concentrations was determined to be negligible for this instrument.

[3] This linear expression is valid only if the virial coefficients are negligible. Owing to the instability of the dodecameric and hexameric forms at pH 10.3, it appears that the magnitude of the virial coefficients must be obtained or estimated by kinetic measurements. A simple calculation (Tanford, 1967, eq 14-24) suggests that in order for the coefficients to be significant, the net charge would be unreasonably large for the hexameric intermediate, though not necessarily so for the dodecamer. Were the virial coefficients for the hexamer significant, the decay kinetics for the next species would not show the observed single exponential decay. Our further neglect of the virial coefficient for the dodecamer is supported by: (a) the nonobservance of an initial lag-phase in the kinetics which would be expected for a large positive coefficient, and (b) the invariance of the kinetics over a twofold concentration range. The latter observation requires that the rate constants reported here cannot differ from the true values (based on nonzero virial coefficients) by more than one standard deviation.

Table I: Rate Constants for CO-, Oxy-, and Methemoglobin Dissociation at Three Temperatures According to a Biphasic Model (Model 1).[a]

Temp (°C)	No. of Residual Runs	k_1 (sec^{-1})	k_2 (sec^{-1})	SSQ[b]
		HbCO Dissociation		
28.8	4	2.9 ± 0.1	0.143 ± 0.006	4.4×10^{-3}
21.0	4	0.71 ± 0.02	0.044 ± 0.001	4.8×10^{-4}
13.1	4	0.17 ± 0.01	0.0080 ± 0.0002	1.8×10^{-3}
		HbO_2 Dissociation		
28.0	5	1.19 ± 0.08	0.0470 ± 0.0003	3.1×10^{-3}
21.0	4	0.26 ± 0.01	0.011 ± 0.0004	3.8×10^{-4}
12.8	4	0.118 ± 0.004	0.0050 ± 0.0001	1.2×10^{-4}
		Met Dissociation		
28.0	5		19.3 ± 0.6	1.0×10^{-3}
21.0	5		5.15 ± 0.09	1.2×10^{-3}
12.8	9		1.66 ± 0.02	4.7×10^{-4}

[a] Twenty data points in each case were used for the parameter fits. [b] Sum of squared residuals (observed vs. calculated) for 20 data points where the initial Y values were 1.8, 1.7, and 0.87 for oxy-, CO-, and methemoglobin, respectively.

Table II: Activation Energies for Three Forms of the Hemoglobin Determined from Rate Constants in Table I.

Ligand Form	Activation Energy[a] (kcal/mol)
HbO_2	26 ± 6
HbCO	32 ± 2
Met	27 ± 3

[a] Activation energies are for the temperature range 12.8–28.8°C. The HbO_2 and HbCO activation energies are for the dodecamer to hexamer dissociation. Similar activation energies were obtained for the overall slow phase of the reaction.

The dissociation curve is biphasic and has approximately 50% slow component. The percent slow component varied slightly in different preparations of the hemoglobin. Since the slow component was sometimes greater than (but never less than) 50% this may indicate that in a few preparations some of the hemoglobin at pH 7 was in the hexameric (Hb_6) form. The theoretical fit and actual data for model I are shown in Figure 1.

The methemoglobin not only had a very different dissociation rate, but also appeared to have only half the overall light scattering change. The dissociation was monophasic, which is consistent with the model $2Hb_6 \rightarrow 12Hb$. When oxyhemoglobin (pH 7) was reacted with ferricyanide in the light scattering stopped-flow to give methemoglobin, a decrease in light scattering corresponding to 50% of the initial signal was observed. The experimental curves for CO, oxy- and methemoglobin dissociation are shown in Figure 2.

The CO-hemoglobin dissociates about four times faster than the oxyhemoglobin, and the met dissociation rate is about 20 times faster than the initial oxyhemoglobin dissociation rate but, perhaps more relevant for comparing dissociation rates of hexamers to smaller forms, the met rate was respectively 100 times and 500 times faster than for the assumed hexameric CO- and oxy-hemoglobins.

The activation energies for the dissociation process of CO-, oxy-, and methemoglobins are given in Table II.

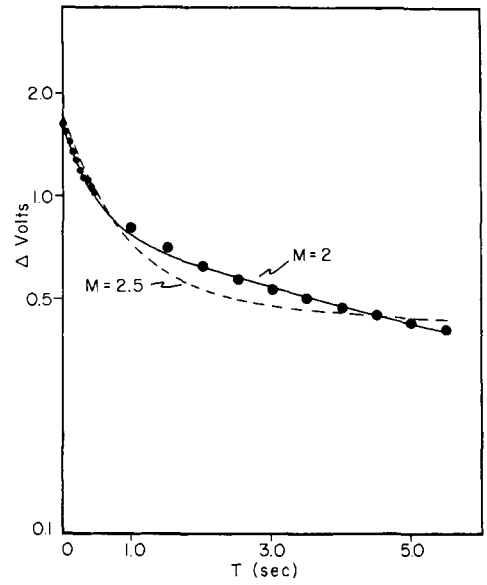

FIGURE 1: Model 1 and model 1a theoretical fits to HbCO data at 28.8°C. (- - -) Model 1a fit where $M = 2.5$; (—) Model 1 fit where $M = 2$. The dots indicate the experimental data points and the size, the standard deviation for the individual points.

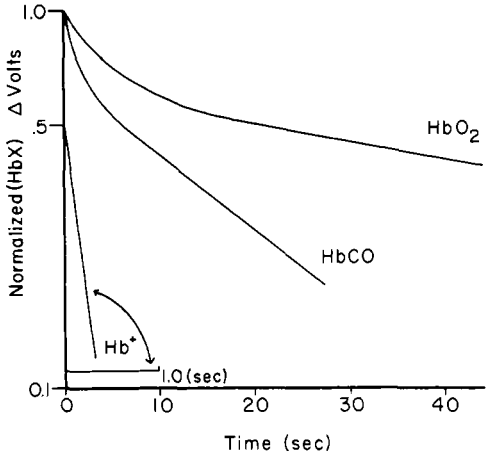

FIGURE 2: Experimental data for oxy-, CO-, and methemoglobin dissociation at 21°C.

Qualitative ultracentrifuge runs indicate that the dissociation process is largely reversible for the CO-bound hemoglobin. When the CO-hemoglobin was incubated for 10–15 min at pH 10.3, the pH lowered to 7, and then centrifuged at 85000*g* for 2.25 hr, 85–90% of the hemoglobin formed a pellet.

Discussion

All three sequential models used to fit the data involved several assumptions. The first assumption was that the dodecamer, Hb_{12}, was homogeneous. Hemoglobin from *Lumbricus* has been shown to give a single band on isoelectric focusing (Rossi-Fanelli et al., 1970). The second assumption was that the reverse reaction could be neglected. Since only the $1/12$th subunits or smaller are observed at pH 10.3 with the electron microscope and since different concentrations of the hemoglobin give the same reaction curves, this was a reasonable assumption, and one which yields relatively simple kinetic equations. The third assumption was that the first splitting of the molecule resulted in two hexamers. Support for this assumption derives from evidence from

electron microscopy which shows cleavage perpendicular to the plane of the ring of the dodecamer and the appearance of hexameric fragments. Furthermore, methemoglobin is apparently stable as a hexamer at pH 7.

In order to determine the sensitivity of the models to variations in molecular weight of the intermediate, and to examine the consequences of the third assumption, model Ia was examined. A fit of the data with model Ia where M = 2.5 is shown in Figure 1. The Fletcher–Powell program would not converge for M = 3 ($Hb_{12} \rightarrow 3Hb_4 \rightarrow 12Hb$). The response function surface was so flat that gradient search methods were not successful in finding a minimum. An attempt was also made to fit the M = 3 case with a "stretching" grid search program (Becsey et al., 1968). The initial grid size was chosen to be ±30% of the initial parameter. Convergence of this program unfortunately gave negative values for k_2. Model Ia was also programmed so that M could be a variable (the molecular weight being $1/M$ that of the dodecamer). In this case, M was found to be 1.89 ± 0.04, very close to the model I value of 2. This fit gave the same sum of squared residuals as model I. This clearly indicated that the light-scattering changes would be very sensitive to the molecular weight of the first intermediate in a sequential kinetic scheme.

The constraints in the models should be noted. In model I, the simple sequential model, the "R_t" expression can be represented as the sum of two exponentials. The constants multiplying the exponentials, which are the relative amplitudes of fast and slow components, are not arbitrary, for their ratio is determined by the ratio of k_1/k_2, and for model I is: $A_1/A_2 = 1.2\text{–}2.2k_2/k_1$. Further support for the hexameric intermediate is thus afforded by the excellent fit by model I to *both* the CO and O_2 data at all three temperatures. The high activation energy for the reaction $Hb_{12} \rightarrow 2Hb_6$ (32 kcal/mol) with cleavage perpendicular to the hexagonal planes would correspond in these models to the activation energy for the breaking of at least four protomer–protomer contacts of about 8 kcal/mol per contact. The activation energy data are not sufficiently precise to distinguish among oxy, CO, and met forms of the protein.

The dissociation also shows a surprising degree of ligand dependence. This ligand dependence cannot be explained solely by differences in spin state since oxy- and CO-hemoglobin are both low spin and methemoglobin is in the low spin state after the pH jump to 10.3. Differences in subunit interaction could derive from protein–heme contacts if there were differences in heme ruffling for the CO and O_2 liganded forms, and/or from ligand–protein interaction if the O–C–Fe and O–O–Fe bond angles and distances were appreciably different. Unfortunately, bond angle data bearing on the latter point do not yet appear sufficiently precise (Lang and Marshall, 1966; Caughey, 1969).

Several attempts were made to observe the association reaction following a pH drop from 10.3 to 7 on samples of dissociated hemoglobin known to associate. When the concentration was 60 μM (heme basis, after mixing) association occurred within the dead-time of the instrument. When the concentration was 10 μM, approximately the last 15–20% of the reaction could be observed. These findings are of considerable interest in regard to the previous kinetic studies of ligand binding following rapid pH changes (Wiechelman and Parkhurst, 1972) which showed that the kinetic properties within 10 msec of mixing were those of the hemoglobin prepared at that final pH and studied 15 min later. Thus, the hemoglobin studied following the pH drop from 10.3 was associated and not protomeric. On the other hand, the previous pH jump kinetic results, when combined with the data reported here, show that the pH 10.3 oxygen dissociation kinetic properties can be assigned to the dodecamer and hence when referenced to pH 7, are to be attributed to pH effects rather than to dissociation. It thus appears that following a rapid pH jump to 10.3, the kinetic properties of the dodecamer change, cooperativity (judged from k, k^* (Wiechelman and Parkhurst, 1972)) largely vanishes and then dissociation occurs.

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