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OXYGEN BINDING OF THE HEME-CONTAINING SUBUNITS OF THE EXTRACELLULAR HEMOGLOBIN OF LUMBRICUS TERRESTRIS

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SUMMARY

Fully oxygenated heme-containing subunits of the extracellular hemoglobin of Lumbricus terrestris were isolated by gel filtration in 0.05 M sodium borate, pH 9.0 at 7 ± 1°C. The polypeptide chain composition of the isolated subunits was determined by SDS PAGE. Most of the subunits exhibited reversible oxygenation curves with oxygen affinities higher than the intact hemoglobin. The Hill plots were nonlinear for Lumbricus hemoglobin and its subunits: the latter exhibited substantial cooperativities as evidenced by Hill constants at half-saturation in the range 2.0 to 2.8.

INTRODUCTION

The annelid extracellular hemoglobins are giant molecules possessing a sedimentation coefficient of about 60S, a hexagonal bilayer appearance in electron micrographs, oxygen affinities generally higher than those of vertebrate hemoglobin and Hill constants ranging from about unity to as high as 5-6 (Mangum, 1976; Weber, 1978; Chung and Ellerton, 1979; Terwilliger, 1980). Lumbricus terrestris hemoglobin is the best studied of these molecules: it possesses a molecular mass of 3.8 to 4.0 x 10^6 by sedimentation equilibrium (Vinogradov et al., 1977; 1980a) and by low angle x-ray scattering (Pilz et al., 1980) and displays a Hill constant of 3.5 to 4.0 at half-saturation (Giardina et al., 1975). It consists of at least six polypeptide chains (Shlom and Vinogradov, 1973) and dissociates at pH above 8 into several subunits (Chiancone et al., 1972: Vinogradov et al., 1977).

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We have used gel filtration of Lumbricus hemoglobin at pH 9.0 to isolate several fractions comprising heme-containing subunits in fully oxygenated form and have determined their oxygen-binding curves at pH 9.0.

MATERIALS AND METHODS

Lumbricus terrestris hemoglobin was prepared as described earlier (Shlom and Vinogradov, 1973). It was stored at $0^{\circ}C$. The hemoglobin was dialzyed for 24 h against 0.05 M sodium borate buffer, pH 9.0. Loads of ca. 240 mg were subjected to gel filtration on a 5 x 95 cm column of Sephacryl S-200 equilibrated with 0.05 M sodium borate buffer, pH 9.0 and maintained at $7 \pm 1^{\circ}C$, at flow rates of 10-20 ml/hr. Fractions of 7 ml were collected and the absorbance of the eluate monitored at 280 nm and 415 nm.

The pooled fractions were concentrated by ultrafiltration using an Amicon UM05 membrane. The absorption spectra was measured on a Cary model 15 spectrophotometer. The oxygenation curves were determined at pH 9.0 using an Aminco Hem-O-Scan analyzer, in which the PO_2 was monitored by a Clark oxygen electrode and the fraction of oxyhemoglobin was measured by dual wavelength spectrophotometry at 560 and 576 nm or at 439 and 448 nm. The partial pressure of O_2 at 50% saturation (P_{50}) was obtained directly from the plots of fractional O_2 saturation versus PO_{50}. The gas mixtures, obtained from Lif-O-Gen, were 25% O_2-75% N_2 for oxygenation and 100% N_2 for deoxygenation. The performance of the Hem-O-Scan analyzer was checked using freshly prepared human hemoglobin.

Polyacrylamide gel electrophoresis (PAGE) was performed using 7.5-20% acrylamide gradient gel slabs, 2 mm. thick, in a Hoeffer apparatus employing the Laemmli buffer system (King and Laemmli, 1971). Samples were incubated in 1.0% SDS at $100^{\circ}C$ for 3 min and electrophoresed in the absence and presence of 2 -mercaptoethanol. The gels were stained with Coomassie brilliant blue R250 and destained in aqueous acetic acid (7.5%)-methanol (25%) solution.

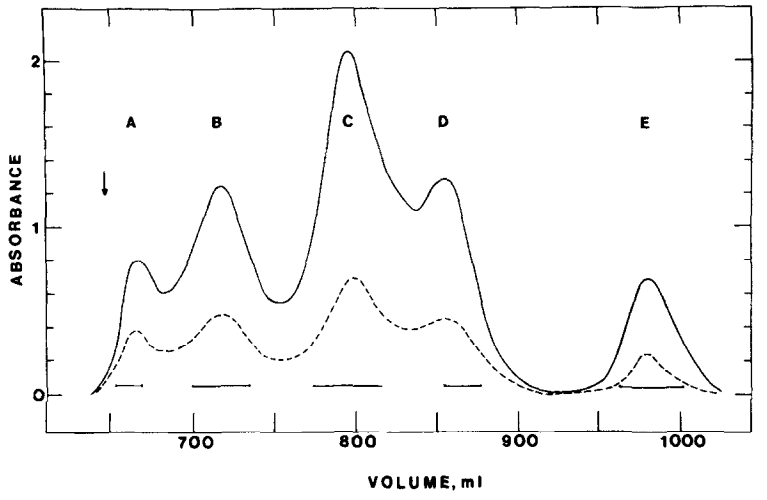

Figure 1. The elution profile at 280 nm (---) and 415 nm (—) of Lumbricus hemoglobin on a 5 x 95 cm column of Sephacryl S-200 in 0.05 M sodium borate pH 9.0 at 7 ± 1°C. The arrow indicates the void volume of the column and the solid bars show the fractions pooled.

RESULTS AND DISCUSSIONS

The elution profile of the Sephacryl S-200 gel filtration is shown in Figure 1 and the SDS PAGE of the resulting fractions is shown in Figure 2. The visible absorption spectra of the fractions corresponding to the five peaks in Figure 1 were all similar to that of Lumbricus oxyhemoglobin and characteristic of oxyhemoglobin spectra in general (Antonini and Brunori, 1971). Methemoglobin formation was less than 10%. SDS PAGE of the peak A and peak B fractions showed that they contain all six polypeptide chains (I-VI) of the native hemoglobin (Kapp and Vinogradov, 1980). The peak C and peak D fractions contain the 50,000 subunit, a disulfide bonded trimer of subunits II, III, and IV, with molecular masses of 14,000, 15,000 and 19,000, respectively (Shlom and Vinogradov, 1973) and subunits V and VI in different proportions. Peak E consists of subunit I, which is known to possess a molecular mass of 16,000 and one heme group (Vinogradov et al., 1977).

Typical oxygenation data obtained at pH 9.0 is presented as Hill plots in Figure 3, for the intact hemoglobin at neutral pH and after incubation at

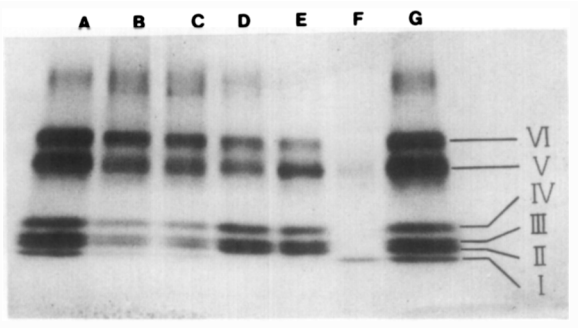

Figure 2. Laemmli SDS PAGE of Lumbricus hemoglobin (A,G) and of its subunits obtained by gel filtration at pH 9.0, peaks A(B), B(C), C(D), D(E) and E(F).

pH 9 and four of the five fractions obtained by gel filtration: peak A, B, C, and E. The oxygenation of all the fractions was reversible. Because the peak D fractions were easily oxidized, we were unable to obtain satisfactory oxygenation curves. The Hill constants at half-saturation and the P_{50} values are given in Table 1. The Hill constant h of our Lumbricus hemoglobin is

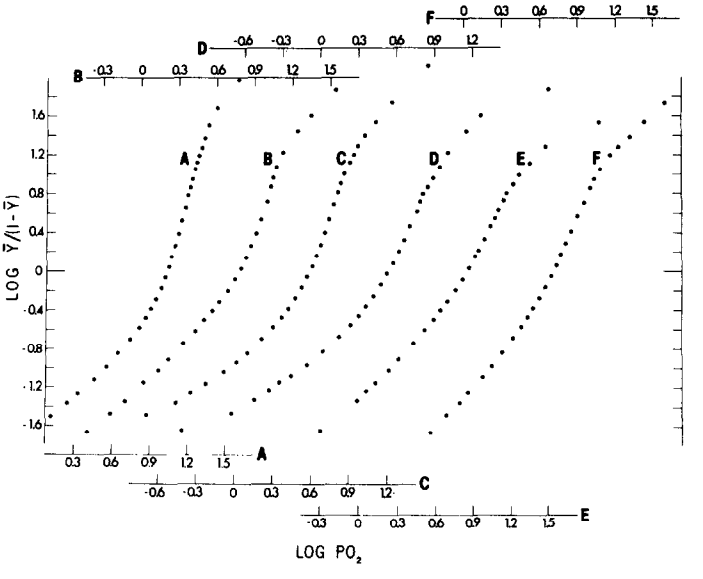

Figure 3. Hill plots of Lumbricus hemoglobin at pH 7.2 (A) and pH 9.0 (B), and of its subunits obtained by gel filtration at pH 9.0, peaks A(C), B(D), C(E) and E(F).

Table 1. Hill constants and P_{50} of Lumbricus Hemoglobin Fractions at pH 9.0.

	P_{50}	h	Subunits
Hemoglobin at pH 7.2	10.9	3.8	I-VI
Hemoglobin at pH 9.0	6.1	2.3	I-VI
Peak A	3.9	2.7	I-VI
Peak B	3.5	2.2	I-VI
Peak C	4.6	1.4	II-VI
Peak D	-	-	II-VI
Peak E	5.2	2.7	I

similar to that obtained by Antonini's group (Giardina et al., 1975), although the P_{50} is appreciably higher.

A characteristic feature of the Hill plots shown in Figure 3 is their nonlinearity: there is a continuous increase in h with increase in oxygen saturation, until about 90%. Hill plots of the oxygen binding of Lumbricus hemoglobin determined tonometrically appear to consist of three linear regions: below 45%, 45 to 80% and above 80% oxygenation (Giardina et al., 1975). The Hill plot obtained in the present study (curve A, Fig. 3), is nonlinear over the whole experimentally available range of oxygenation of the hemoglobin. Such nonlinear Hill plots with the maximum value of h attained at 80 to 90% oxygenation have also been obtained with fresh Lumbricus and Arenicola blood as well as Arenicola hemoglobin (Kapp and Vinogradov, unpublished observations).

An irreversible decrease in h from 3.8 to 2.1-2.3 occurs upon exposure of Lumbricus hemoglobin to changes in pH and temperature (Giardina et al., 1975). It was suggested that the native hemoglobin with h ≃4 is in a "metastable" conformation which can be converted irreversibly into a "stable" form of lower cooperativity. We also observed a decrease in the cooperativity of the intact Lumbricus hemoglobin from 3.8 to 2.9 after storage at $0^{o}C$ (Kapp

and Vinogradov, 1980). A similar phenomenon was recently observed in the case of Eisenia fetida hemoglobin (Frossard, 1981). The nature of this conversion has not been elucidated; circular dichroism studies have shown that the decrease in cooperativity is accompanied by alterations in the heme environment (Ascoli et al, 1976). The cooperativity of the "stable" form of earthworm hemoglobin (Ascoli et al., 1976) is apparently the same as the cooperativities of the 9-10S subunits of the earthworm hemoglobin obtained by dissociation of alkaline pH (David and Daniel, 1973), i.e., about 2.1 to 2.4.

The unexpected finding in the present work (Table 1) is that most of the fractions isolated by gel filtration exhibit substantial cooperativity in their oxygen binding and oxygen affinities higher than the hemoglobin at neutral or alkaline pH. A complicating factor is that these fractions are physically inhomogenous (Vinogradov et al., 1977; Kapp and Vinogradov, 1980). The SDS PAGE polypeptide chain composition of the peak A fraction differs from that of the hemoglobin and of the 9-10S subunit obtained by reassociation at neutral pH (Kapp and Vinogradov, 1980): thus, it probably is not a 1/12th subunit of the native hemoglobin. The cooperativities of the peak B and peak C fractions which consist predominantly of the 50,000 subunits are similar to those of the "stable" conformation of Lumbricus hemoglobin and of the peak A fraction. The 50,000 subunit and subunit I represent 60 $\pm$ 10% and 20 $\pm$ 5% of the whole molecule, respectively, according to the results of SDS PAGE (Shlom and Vinogradov, 1973) and of PAGE at pH 9.0 (Vinogradov et al., 1977; Vinogradov et al., 1980b). The fact that about 90% of the heme groups in the hemoglobin are carried by two different subunits is supported by the results of recent kinetic studies (Giacometti et al., 1975). The fractions consisting of peak B and C probably represent several fragments of Lumbricus hemoglobin with overlapping molecular mass distributions. Peak E consists of a single polypeptide chain of 16,000 but on gel filtration at alkaline pH its elution volume corresponds to a molecular mass of 25,000 to

30,000 suggesting that it aggregates at pH 9.0 (Vinogradov et al., 1977). So far we have not been able to isolate subunits V and VI in pure form and hence do not know whether they aggregrate as well. The effect of aggregation on the oxygenation of the subunits of Lumbricus hemoglobin obtained by gel filtration at alkaline pH remains to be elucidated.

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