ELSEVIER

The Spontaneous Hemin Release from *Lumbricus terrestris* Hemoglobin

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ABSTRACT. The slow, spontaneous release of hemin from earthworm, *Lumbricus terrestris*, hemoglobin has been studied under mild conditions in the presence of excess apomyoglobin. This important protein is surprisingly unstable. The reaction is best described as hemin released from the globin into water, followed by quick engulfment by apomyoglobin. The energetics of this reaction are compared with those of other types of hemoglobins. Anomalously low activation energy and enthalpy are counterbalanced by a negative entropy. These values reflect significant low frequency protein motion and dynamics of earthworm hemoglobin and may also indicate an open structure distal to the heme. This is also supported by the infrared spectrum of the carbonyl hemoprotein, which indicates several types of distal interactions with the bound CO. The reported low heme to polypeptide ratio for this protein may be due to facile heme and hemin release by the circulating protein. COMP BIOCHEM PHYSIOL 118A;4:1241–1245, 1997. © 1997 Elsevier Science Inc.

INTRODUCTION

The circulatory hemoglobin of earthworm is present as a free protein, in contrast to vertebrates where Hb is contained within the erythrocyte membrane, and is much less protected from interactions with the vascular wall and foreign agents. This protein has a high molecular weight, 3.9 to 4.4 MDa, is a multimeric protein composed of at least five different subunit types, some of which do not appear to bind heme (5,20). These are arranged in self-organized and self-limiting fashion into a gigantic protein containing about 220 subunits (11,13). The large size of these extracellular oxygen carriers is thought to be necessary; Hbs of smaller mass would force the osmotic pressure higher than the organism could bear. These large Hbs exhibit high ligand-binding cooperativity with n_{50} from 3.3 for *L. terrestris* (18), up to 9.5 for *Eisenia foetida* and *Pheretima hilgendorfi* Hbs, depending upon pH, Ca^{2+} and Zn^{2+} concentrations (6,8,9). In place of chloride ion and sugar phosphates, which are effectors of mammalian and avian Hbs, pH and dibasic cations appear to play the roles of allosteric effectors

for these Hbs. Isolated earthworm Hb appears to be an unstable hemoprotein which rapidly releases hemin into the solvent.

Free hemin and globin cannot be tolerated by higher vertebrates and can lead to a variety of complications through increased Heinz body formation including hemolytic anemia (1). It is known that the heme in Hb is rapidly oxidized to hemin by a range of drugs and common chemicals including phenols, hydrazines, and nitrites with clinical complications (7). Hemin is also continuously formed in the blood of apparently healthy individuals as a product of chloride assisted oxidation of hemoglobin at a rate of 1–2% daily (19). Using apoMb as the hemin sink, we have shown that the spontaneous release of hemin from the β chains of human Hb and single point mutants is a continuous process with a half-life of only a few hours (14). This system measures only the hemin release and is independent of hemin apoMb combination (4).

Hemin transfer from an intact donor protein to an acceptor protein is illustrated in a reaction triangle in Fig. 1. Some donor- or holoproteins can be observed in two forms: a form that displays the normal, native activity and a form that displays much less enzymatic activity. The barrier for hemin release is measurably lower for the inactive form of hemoprotein than from the active, but slower hemin releasing holoprotein. The active and inactive holoforms occupy the two left corners of the transfer triangle. The equilibrium between these two forms of holoprotein can be perturbed
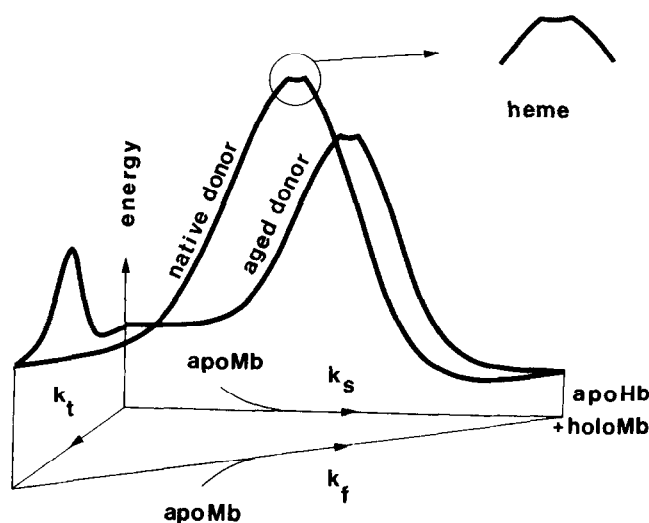
FIG. 1. Diagrammatic relationship between the protein species and energies of hemin release. Note that the energy of the products, at right, are only slightly lower than the energy of the reactants, at left. The activation energy, at the top, is many times larger than the overall reaction energy.

by changes in temperature, prolonged aqueous storage or addition of certain ligands to the system. Addition of apo-myoglobin to the system moves the equilibrium toward the third corner, with production of holoMb and apodonor protein. It was previously shown that the hemin leaves the donor protein during spontaneous fission and the apoMb does not interact with the donor protein (15). The observed energetics are, therefore, characteristic only of the donor protein. We have observed at least two forms of earthworm Hb, both rapidly undergo fission under mild conditions, exhibiting relatively low activation energies and enthalpies. The rate of hemoprotein fission is greater for the oxidized form of earthworm hemoglobin than for any other protein so far observed.

EXPERIMENTAL

About 100 earthworms were washed in water, decapitated and the blood squeezed into a beaker containing a few ml of standard buffer; 50 mM phosphate, 1 mM EDTA, pH 7.0. The mixture was centrifuged, filtered then concentrated to 1.5 mM heme under nitrogen. Part of this preparation was gassed with CO for 15 min, injected into an IR cell with CaF_2 windows and the IR spectrum collected on a DIGI-LAB 15/90E spectrometer fitted with a liquid nitrogen cooled detector. Citrated blood from a domestic rabbit was centrifuged and washed several times with isotonic saline. The packed red blood cells were gassed with CO for 15 min before injection into the IR cell. The data were resolved into component curves using the program SAAM II, courtesy of the University of Washington. The remainder of the earthworm Hb was chromatographed down a 40 × 1 cm

column of G-25 Sephadex® (Pharmacia) equilibrated with standard buffer. This portion was frozen in liquid nitrogen, as beads, until further use. Earthworm hemoglobin can be chromatographed after thawing with no observable insoluble residue and the elution profile is consistent with high molecular weight and little subunit dissociation.

A typical kinetic experiment was begun by thawing a few beads of the frozen Hb, while the buffer was incubated in a thermostated quartz cuvette in a Beckmann DU-7 spectrophotometer. The apoMb was separately incubated for 15–20 min in a water bath. A small amount of the earthworm Hb was added to the buffer, then heme was always oxidized to hemin by addition of a single grain of $K_3Fe(CN)_6$. The reaction was initiated by addition of a small amount of apoMb (0.5 mM) and the formation of holoMb followed at 409 nm overnight, which was at least three half-lives. Some reactions were followed by repeated scanning from 370 to 450 nm; isosbestic points were observed at 391 and 425 nm. From 25 to 52 data pairs were collected for each determination and the data were analyzed using the programs SYS-TAT® and RS/1®. A portion of the earthworm hemoglobin was aged by refrigeration in standard buffer from 10 to 14 days. Rabbit maintenance, procedures and experimentation were conducted in conformity with humane guidelines of Sweden.

RESULTS

The infrared spectrum of carbonyl earthworm Hb exhibits a strong band with a maximum at 1951 cm^{-1}, which is the energy commonly observed for COHbs (12). This spectrum can be resolved into at least four bands, three of these being of lower energy and strength than the primary band at 1951 cm^{-1}, Fig. 2. The band width, at half height, of the primary band at 1951 cm^{-1}, is 23 cm^{-1}, which is much broader than observed for most other Hbs, which exhibit narrow bands, 8 cm^{-1}, at 1951 cm^{-1} (12). Much smaller bands were observed at 1936, 1913, and 1903 cm^{-1}, though this lowest energy absorbance is very small and may not be real.

The hemin release data were best fit as either a single first order or two simultaneous first order hemin transfers, by both programs. Close inspection of the plotted data revealed three or four simultaneous reactions of very slightly different rates, however the curve fitting procedures used did not give us greater confidence in results from a multiple first order fit than the simple fit of a single first order reaction, so the single rate constant is used in discussion. In addition, a slow, linear absorbance increase was observed at the end of the experiments which was presumably due to precipitation of some apoHb and made estimation of the end absorption difficult, as has been observed for human Hb (14). This slow reaction was not further studied.

The overall rate constant at 298°K, 1.9×10^{-3} min^{-1}, was larger than for any other native protein so far studied and about twice as fast as hemin release from human Hb A
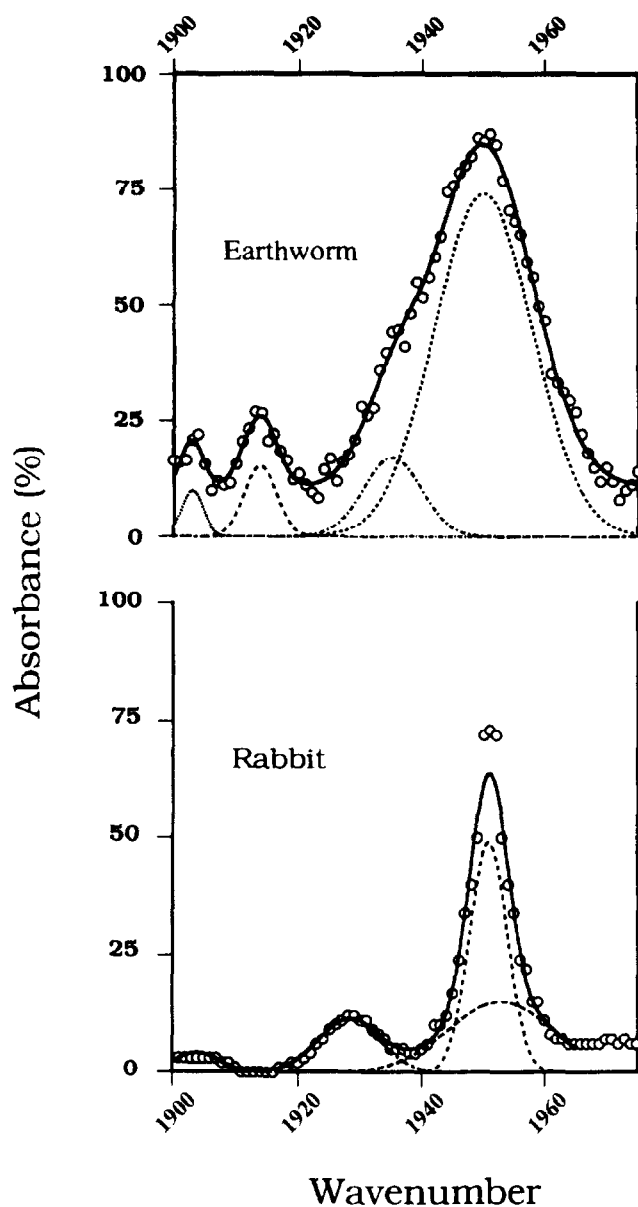
FIG. 2. Infrared spectra of earthworm COHb, top, and domestic rabbit COHb, in the CO stretch region. The circles are the data points used for curve resolving. The dashed lines are the resolved curves and the solid lines represent the linear combinations of the resolved curves.

(Table 1). The Ahrrenius plots of the release rates as a function of inverse temperature are shown in Fig. 3. The activation energy for the isolated earthworm Hb is 89 ± 9 kJ mol^{-1} and only 50 ± 10 kJ mol^{-1} for the aged protein, both being much lower than fission of Hb A β chains, 124 kJ mol^{-1}. These low activation energies are counterbalanced by negative activation entropies. The free energies of activation, $\Delta G^{\circ\ddagger}$, were calculated as 93 and 91 kJ mol^{-1} for the freshly isolated and aged proteins, respectively, which is insignificantly lower than observed for Hb A β chains or legume Hb. This separation of free energy into enthalpy and

entropy is strictly valid only for rigid potentials and care should be taken with interpretation of these quantities.

DISCUSSION

The IR spectrum indicates the binding site for gaseous ligands in earthworm Hb is little different from other COHbs, consistent with a proximal histidyl (ligand 5) to the heme Fe(II) and a distal histidyl group neighboring the CO for most subunits. The minor CO stretch at 1936 cm^{-1} is consistent with distal histidyl substitution by glutamyl, as in the opossum (*Didelphis marsupialis*) with γCO at 1943 cm^{-1} or with a distal situation similar to that of domestic rabbit with γCO at 1929 cm^{-1}, possibly caused by a "tighter fit" of this distal group with the distal histidyl (12). The CO stretches of peroxidases with histidyl as L5 and histidyl and arginyl residues distal to the Fe(II) are observed from 1938 down to 1903 cm^{-1}, some of the CO stretch(es) of CO cytochrome P450 with a cystidyl L5 are observed at lower energies than COHb (10). The absence of significant absorbance between 1960–1970 cm^{-1} indicates that the protein surrounding the heme was primarily in the native conformation; CO hemoproteins from proteins reconstituted with heme and containing some nonnative hemoprotein, almost invariably exhibit a band in this region despite chromatography to remove excess hemin (16), denaturation also results in absorbance between 1960–1970 cm^{-1} (10).

Table 1 also presents the results from studies of the hemoprotein fission of several oxygen carriers and peroxidases, which exhibit a $\Delta G^{\circ\ddagger}$ about 10 kJ mol^{-1} larger than any oxygen carrier. We suggest that this difference is due to the difference in water coordination in the heme pocket. The highly polar groups present in the pocket of peroxidases appear to control the water(s) in a manner that both increases electron availability at iron and discourages hemoprotein fission. It is interesting that the earthworm Hb exhibits the shortest half life for protein fission of any native hemoprotein so far examined. There are two possible reasons for this property (a) the flexibility of the polypeptide, with demands for communication between subunits that is required in Hbs for cooperative dioxygen binding (b) the high mass of the Hb molecule which offers more channels for concentrating energy to the heme pocket required for hemin release. Reason (a) is supported by the IR data of 23 cm^{-1} band width for the primary absorbance, which suggests a much more open distal pocket, allowing more facile heme release. There also appears to be a trend towards higher rates of protein fission with increasing molecular weight, supporting reason (b). Perhaps the low frequency dynamics with high kinetic energies are more allowed in high MW proteins. If so, other invertebrate, giant Hbs are probably also relatively unstable.

Previous studies indicated that the energy barrier for hemin release consists of two parts; Fe-L5 bond breakage and hemin solvation (15). The free energies of hemin and por-

TABLE 1. Kinetic parameters for hemin release from *Lumbricus terrestris*, other hemoglobins and a peroxidase

Protein	Temp. range (°C)	Rate constant (min^{-1} × 10^3)	$\Delta H^\circ\dagger$ (kJ/mol)	$\Delta G^\circ\dagger$ (kJ/mol)	$\Delta S^\circ\dagger$ (J/mol.°K)
L. terrestris	14–30	19 ± 4	87 ± 9	93	−22
Aged terrestris	20–37	38 ± 6	48 ± 10	91	−146
Human Hb A	25–42	7.7	120	95	82
Glycine soya (soybean) Hb	>23	7.1	130	95	117
Armoracia rusticana (horseradish peroxidase) C	30–45	0.24	131	104	91

phyrin fission differ by about 10 kJ mol^{-1}, which was assigned to the Fe-L5 bond. The remaining 80–90 kJ mol^{-1} was interpreted being due to the energy required to solvate porphyrin—that is the difference in surface energies of aquated porphyrin and porphyrin buried within the protein. The $\Delta G^\circ\dagger$ found here for the native earthworm Hb is consistent with this interpretation. Facile hemin exchange is the mechanism used to control the activity levels of the proteins tryptophan-2,3-dioxygenase (3), and prostoglandin endoperoxidase synthetase (17). Heme release may not be important for the well-being of L. terrestris, but a continuing problem to be tolerated in exchange for a lowered osmotic pressure and a freely circulating Hb.

The results show that spontaneous release of hemin occurs even from an important protein that freely circulates in blood. The results also show that oxidized earthworm Hb undergoes hemoprotein fission more readily than any other hemoprotein and this rate of self destruction increases dur-

ing storage. This is probably due to a combination of a ligand binding site more open to solvent than other hemoglobins and to greater kinetic energies associated with low frequency molecular motions in giant proteins. This may be a partial explanation for the reported unsaturation of heme binding sites for this oxygen carrier (20).

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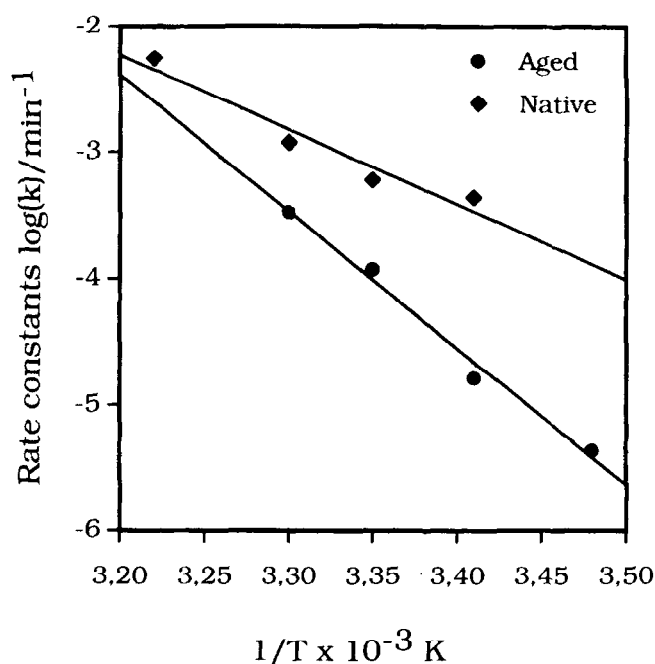
FIG. 3. **Arrhenius plot of the first order rate constants for hemin release from native (♦) and aged (●) earthworm Hb, with the temperature ranges as given in Table 1. The lines shown are the linear best fits.**

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