OXIDATION–REDUCTION POTENTIALS OF HIGH MOLECULAR WEIGHT HEME PROTEINS

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1. Introduction

Previous studies on the oxidation by potassium ferricyanide of the high molecular weight heme proteins of Annelids, erythrocruorins and chlorocruorins, brought out several differences with respect to tetrameric hemoglobins. The nature of the oxidation product depends on the pH of the medium; thus a high molecular weight derivative with the spectral properties of aquo-met-heme proteins can be obtained only at pH values near neutrality. At acid and alkaline pH values a low molecular weight hemichrome is formed. Moreover, complete oxidation is achieved with a stoichiometric amount of ferricyanide only in the case of the deoxygenated derivative; it requires a large excess of oxidant in the case of the oxygenated derivative. In accordance with this observation, the met-form reverts partially into the oxygenated form upon removal of ferro- and ferricyanide by dialysis in the presence of oxygen [1,2]. The latter data pointed to a higher value of the oxidation-reduction potential in erythro- and chlorocruorins with respect to tetrameric hemoglobins.

This communication presents results of anaerobic spectrophotometric oxidation-reduction titrations of erythrocruorins performed in the presence of the ferro-ferricyanide system. In all the proteins studied the redox potentials are around 0.22–0.23 V at pH 7.0 and 20°C, a value which is significantly higher than that of human hemoglobin under similar experimental conditions [3].

2. Materials and methods

The erythrocruorins from *Lumbricus rubellus*, *Nereis diversicolor* and *Octolasium complanatum* were prepared as described previously [4,5].

The protein was dissolved in phosphate buffer I = 0.1 M at pH 7.0 and the samples used within a few days. The concentration was determined using a mM extinction coefficient of 11.8 at 564 nm for the deoxygenated derivative of all the species [5]. Potassium ferricyanide and ferrocyanide were of analytical grade. Their solutions were prepared just before use, by dissolving the salts in phosphate buffer at pH 7.0, I = 0.1 M, and were kept in the dark. The concentrations of ferricyanide and ferrocyanide were determined by titration with sodium thiosulfate, in the presence of iodide, and cerium(IV) ammonium sulfate respectively.

The anaerobic redox titrations were performed in a modified Thunberg vessel. The erythrocruorin solution (typically 3 ml at a concentration of 50 μM) and the ferrocyanide solution (50 μl) were placed in the two arms and carefully deoxygenated by deaeration and equilibration with oxygen-free argon. Subsequently the two solutions were mixed and the visible spectrum recorded. The system was titrated by addition of increasing amounts of an anaerobic ferricyanide solution injected through a rubber stopper. The final concentrations of ferro- and ferricyanide were about 15 mM and 0.1 mM respectively. The extent of the oxidation reaction was measured between 480 and 700 nm in a Cary 219 spectrophotometer. Preliminary experiments indicated that equilibrium is reached within seconds; thus the spectra were recorded a few minutes after each addition of ferricyanide. The temperature was kept at 20°C throughout the experiments using a Haake FK circulating thermostat.

The binding of ferrocyanide to *Lumbricus rebellus*

erythrocruorin was measured in gel filtration experiments [6]. A column (40 × 1 cm) of Sephadex G-25 (fine) was equilibrated with a solution of potassium ferrocyanide at a concentration of 5 mM in phosphate buffer pH 7.0, I = 0.1 M. Then a known amount (0.1 μmol on a heme basis) of oxyerythrocruorin in a solution of potassium ferrocyanide (at the concentration used for the equilibration) was applied to the column. Elution was performed with the same potassium ferrocyanide solution. The absorbance at 340 nm of the column eluent was monitored continuously with a Gilford Model 2000 multiple sample absorbance recorder equipped with flow-through cells. The flow rate was maintained constant at 15 ml/h. Readings at 340 nm were constant except for a peak corresponding to erythrocruorin plus the erythrocruorin-potassium ferrocyanide complex and a trough corresponding to the region from which the ferrocyanide was taken up by erythrocruorin. Since the flow rate was maintained constant, the area of the trough could be related to the amount of potassium ferrocyanide bound to erythrocruorin.

For the difference spectra, two matched cuvettes were filled with the same volume of an erythrocruorin solution at a concentration of 30 μM in phosphate buffer at pH 7.0, I = 0.1 M. Equal volumes of buffer and potassium ferrocyanide (up to a final concentration of 0.0125 M) were added stepwise in the reference and sample cells respectively. The spectra were recorded between 500 and 700 nm on a 0.1 absorbance full range scale.

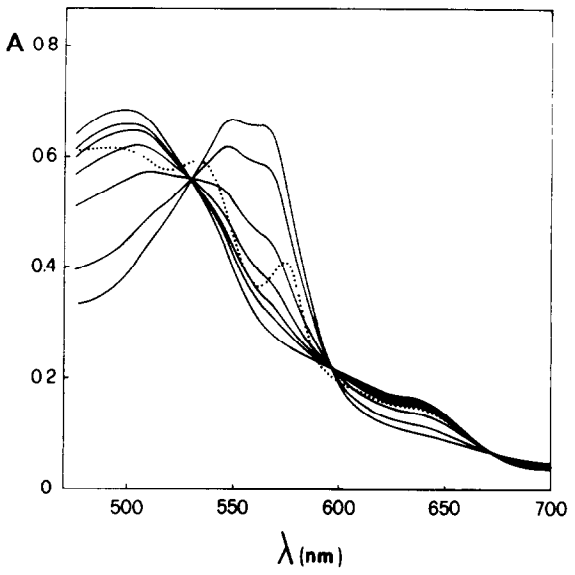

Fig.1. Anaerobic redox spectrophotometric titration of *Lumbricus rubellus* deoxyerythrocruorin in the presence of the ferrocyanide/ferricyanide system. Protein concentration, 30 μM in phosphate buffer, pH 7.00, I = 0.1 M. The dotted curve refers to the spectrum obtained after admission of air in the tonometer. For details see text.

3. Results and discussion

Determination of the redox potential of erythrocruorin was limited to pH 7.0, due to the irreversible formation of low MW hemichromes from met-erythrocruorin outside the neutral pH range. At pH 7.0, the system is fully reversible i.e. met-erythrocruorin can be reduced to yield a deoxygenated derivative with the same physicochemical properties as deoxy-erythrocruorin [1].

A spectrophotometric anaerobic redox titration of *Lumbricus rubellus* erythrocruorin at 20°C is shown in fig.1; the presence of isosbestic points supports the existence of only two erythrocruorin species in solution. The final spectrum is characteristic of an aquo-met heme protein [3] indicating the completeness of the oxidation reaction. Upon admission of air at the end of the reaction, the absorption spectrum revealed formation of some oxygenated derivative (fig.1). This finding was expected because previous observations had shown that a large excess of potassium ferricyanide was required to achieve complete oxidation of oxy-erythrocruorins and *Eudistilia* oxychlorocruorin [1,7].

Control experiments were performed to measure the binding of ferrocyanide to oxyerythrocruorin, since it is known than human oxy-hemoglobin binds ferrocyanide strongly [3]. Gel filtration experiments indicated that 1–1.5 mol of ferrocyanide are bound/mol of oxyerythrocruorin (on a heme basis). Thus, at the concentrations used in the redox titrations, the amount of ferrocyanide bound does not affect its free concentration in solution significantly. Furthermore, no changes occur in the visible bands of the oxyerythrocruorin spectrum when ferrocyanide is added to the solution.

In fig.2 a plot of the redox data of *Lumbricus rubellus* is shown. The same results were obtained using an eight-fold range of concentrations of ferro- and ferricyanide.

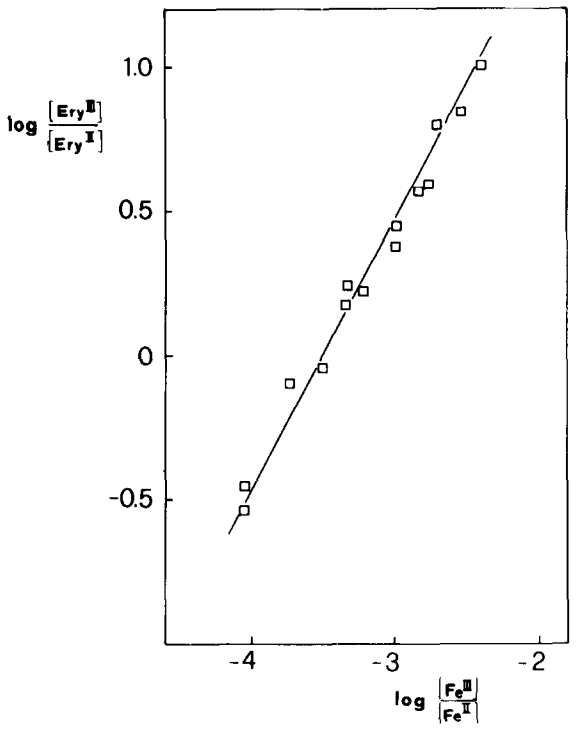

Fig.2. Oxidation-reduction potential of *Lumbricus rubellus* erythrocruorin. Plot of log ($[Ery^{III}]/[Ery^{II}]$) against log ($[Fe^{III}]/[Fe^{II}]$). Protein concentration, 30 μM in phosphate buffer, pH 7.00, I = 0.1 M. For details see text.

The Nernst equation for the reaction:

$$Ery^{II} \rightleftharpoons Ery^{III}$$

can be written as follows for a temperature of 20°C:

$$E = E_{1/2} + \frac{0.0581}{n} \log \frac{[Ery^{III}]}{[Ery^{II}]}$$

where E is the value of the redox potential and $E_{1/2}$ is the potential of the system at the midpoint, when $[Ery^{II}] = [Ery^{III}]$. From the value of $\log \frac{[Fe^{III}]}{[Fe^{II}]}$ at the midpoint and that of the standard redox potential for the ferricyanide/ferrocyanide couple (0.43 V at 20°C, I = 0.1 M) [8], a value of $E_{1/2} = 0.23$ V is obtained. The redox n-value (the slope of fig.2) which is very nearly 1, indicates absence of cooperativity in the oxidation reaction. Under similar experimental conditions, the Hill constant for oxygen binding was found to be 3.2. The absence of cooperativity in the redox reaction of erythrocruorin is at variance with the behavior of hemoglobin where, at neutral pH, cooperativity is only decreased as compared to the oxygenation reaction [3]. In the case of human hemoglobin two types of explanations for the lower value of n in the redox equilibrium have been provided: intramolecular heterogeneity [9,10] and/or presence of a substantial amount of T form in met-hemoglobin [11,12]. Both mechanisms may apply to erythrocruorins.

Table 1
Redox potentials of erythrocruorins

Species	$E_{1/2}$	n
Lumbricus rubellus	0.23	1.0
Octolasium complanatum	0.23	1.0
Nereis diversicolor	0.22	1.1

Phosphate buffer at pH 7.0, I = 0.1 M; temperature, 20°C

Erythrocruorins from other species (*Octolasium complanatum* and *Nereis diversicolor*) have similarly high values of the redox potential and a value of n equal to 1 (table 1). An even higher value of 0.28–0.30 V was obtained for *Spirographis spallanzanii* chlorocruorin at pH 6.8 (Brunori, M., unpublished), suggesting that high oxidation reduction potentials are a general characteristic of high molecular weight heme proteins. The difference with respect to Hb A ($E_{1/2} = 0.14$ V) may be related to structural differences at the heme site in these proteins.

The biological significance of the high redox potential in Annelids may be of importance for maintaining low levels of oxidized protein.

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