EVIDENCE FOR COOPERATIVE EFFECTS IN THE OXIDATION OF DEOXYHEMERYTHRIN BY FERRICYANIDE

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1. Introduction

Hemerythrin is a respiratory pigment of invertebrates in which non-heme iron is involved in the interaction with the ligand, the stoichiometry of binding corresponding to two metal atoms per oxygen molecule [1]. Clear evidence for the presence of reaction intermediates has been obtained in a study of the kinetics of ligand binding by deoxyhemerythrin, which has shown that the relaxation spectrum in the reaction with oxygen involves at least two, widely distinct, relaxation times [2]. The available information on the structure and interactions of the metal atoms in the O_2 binding site, which is still rather incomplete, is mainly obtained from various types of spectroscopic intestigations [3]. In particular, Mössbauer spectroscopy has given indication that the iron atoms, which appear homogeneous in the deoxy or ferric derivatives, give rise to two distinct signals upon oxygen binding [4–6]. Moreover, other measurements by the same authors indicate that the iron atoms in ferric hemerythrin are antiferromagnetically coupled, thereby excluding the possibility of obtaining EPR signals [4].

While most ligands react with hemerythrin in a 1:2 ratio (L/Fe), oxidation of the site involves removal of one electron from each iron atom, as shown below. Thus partial oxidation of the protein may possibly be used as a means of uncoupling the two metal atoms in each site.

This note is preliminary to a study on the oxidation–reduction equilibrium and the kinetics of redox reactions of hemerythrin from *Sipunculus nudus*. The results reported below indicate that oxidation of the iron atoms in a site is a highly cooperative process, since it occurs in an all-or-none fashion.

2. Materials and methods

Hemerythrin was prepared from the worms *Sipunculus nudus* according to the procedure previously described [2]. Immediately after preparation the protein solution was deoxygenated by vacuum and stored in a tonometer under argon at 4°.

Protein concentration was measured from the extinction coefficient of oxyhemerythrin at 500 nm ($E = 1.1 \times 10^3\ M^{-1}\ cm^{-1}$). The following buffer systems were used: 0.1 M potassium phosphate and 0.1 M tris-perchlorate.

The spectrophotometric titrations were carried out using a Cary 14 spectrophotometer equipped with a thermostatted cell compartment. A 1 cm quartz cuvette containing a small glass bead for mixing and closed with a rubber stopper containing

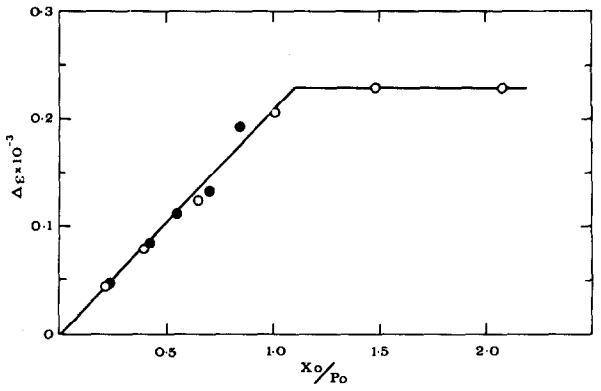

Fig. 1. Stoichiometric titration of deoxyhemerythrin with potassium ferricyanide. The changes in molar absorptivity (ΔE) were measured at 500 nm as a function of the ratio of ferricyanide concentration (Xo) to hemerythrin concentrations expressed as iron (Po). Conditions: •, 11×10^{-4} M hemerythrin; ○, 11×10^{-4} M hemerythrin; buffer 0.1 M phosphate pH 7.0; temperature = 20°.

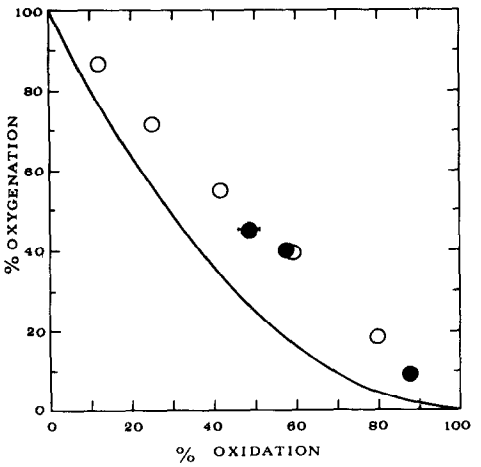

Fig. 2. Relationship between the percentage of oxygenation and the percentage of oxidation. Conditions: •, 2×10^{-4} M hemerythrin in phosphate pH 7.0, 0.1 M; ○, 4×10^{-4} M hemerythrin in tris-perchlorate pH 8.0, 0.1 M; temperature 20°. The continuous line shows the drop in the oxygen combining capacity expected if the oxidation of deoxyhemerythrin occurred in a statistical fashion; it was calculated from a binomial distribution, $(p + q)^2$, on the assumption that only fully reduced sites (p) would be able to combine with oxygen.

a needle for overflow, was degassed with argon and filled successively with deoxyhemerythrin by introducing the needle of a syringe containing the protein through the stopper. A solution of degassed potassium ferricyanide was added with an Agla micrometer syringe and the absorbance change recorded after each addition. Measurements were made at different times to ensure achievement of equilibrium.

EPR spectra were recorded on a Varian 4502-14 Spectrophotometer, equipped with 100 kHz field modulation within a Varian multipurpose cavity. Temperature of experiments was −180° and was obtained with the Varian variable temperature accessory. Field modulation amplitude was about 10 G; microwave power 20 mW. The EPR experiments with deoxyhemerythrin were carried out by freezing the samples immediately after anaerobic transfer of the degassed protein solutions into EPR tubes which had been flushed with argon for 5 min.

3. Results

The spectral changes accompanying the addition of ferricyanide to deoxygenated hemerythrin show a complex pattern*. In the region between 500 and 650 nm, a fast increase in optical density, which at millimolar concentrations of ferricyanide occurs in the millisecond time range, is followed by a very slow decrease in optical density, with a half time of several minutes. The relative contribution of the slower phase is larger at some wavelengths (e.g. 600 nm) than at others (e.g. 500 nm). After a suitably long time (10 to 30 min depending on the experimental conditions), equilibrium is attained, as indicated by the steady value of the optical density.

Fig. 1 reports the change in extinction at 500 nm, observed at equilibrium after addition of ferri-

* The mechanism of electron transfer in hemerythrin appears very complex, and is presently under investigation. At present, no clear interpretation of the various phases in the kinetics of oxidation of hemerythrin can be offered.

cyanide to deoxyhemerythrin, as a function of the molar ratio of oxidant to iron. The end-point of the titration closely corresponds to a stoichiometry of one ferricyanide per iron atom, showing that all metal atoms in deoxyhemerythrin are stoichiometrically oxidized by ferricyanide. Similar results were obtained starting from oxyhemerythrin, the stoichiometry of oxidation corresponding, in this case as well, to a 1:1 ratio, in agreement with previous observations [6]. On the basis of these results two sets of experiments were performed, starting in all cases from deoxyhemerythrin.

(a) After addition of a given amount of ferricyanide to a known amount of deoxyhemerythrin, the optical density change was followed to equilibrium; this allowed us to check the stoichiometry of oxidation under the conditions used. Thereafter the solution was exposed to air, and the optical density change following combination with oxygen of the sites still capable of binding the ligand was measured; this optical density change was taken to indicate the residual oxygen-combining capacity of the protein.

The relationship between fractional oxidation of the iron atoms and fractional oxygen binding capacity, obtained in this type of experiments, is shown in fig. 2. The continuous line represents the drop in the ogygen-combining capacity expected if the oxidation of the iron atoms by ferricyanide occurred in a purely statistical fashion; it was calculated by the use of a binomial distribution, assuming that a site with mixed oxidation state (i.e. Fe^{+2}–Fe^{+3}) is incapable of combining with oxygen. The assumption appears to be supported by the presence, at intermediate oxidation states, of only two spectroscopically distinct species, corresponding to fully reduced and to fully oxidized protein.

(b) EPR spectra of the partially oxidized, fully oxidized and reduced hemerythrin were measured in parallel experiments. In all cases, apart from a very small signal reflecting spurious iron, no detectable resonances were observed under any condition. It is relevant to point out that in the specimens corresponding to partial oxidation, EPR spectra were taken, with identical results, either freezing the solution immediately after addition of ferricyanide, or allowing the sample to reach equilibrium at 20° before freezing.

4. Conclusions

The experiments reported above allow the following conclusions concerning the redox properties of hemerythrin to be drawn:

(i) Ferricyanide is capable of oxidizing stoichiometrically all iron atoms in deoxyhemerythrin, as indicated by the titration experiment reported in fig. 1. This sets a minimum value for the redox potential of hemerythrin, which must be considerably lower than that of the ferri-ferrocyanide couple (ΔE minimum $\sim$ 150 mV). The data also indicate that, at equilibrium, only two spectroscopic species are present throughout the titration.

(ii) The relationship between fractional oxidation and fractional oxygen combining capacity (fig. 2) shows that, within any site, oxidation of the two iron atoms occurs in a highly cooperative fashion, and oxidation intermediates are destabilized in respect to the fully reduced or fully oxidized forms. The EPR experiments indicate that even in the absence of oxygen, species having only one iron atom per site in the ferric form are not found in partially oxidized solutions. Thus oxidation of the iron atoms in each site is a cooperative process independent of coupling effects which, in the presence of ligands such as oxygen, may arise from the special stoichiometry of binding.

References

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