THE OXYGEN EQUILIBRIUM OF YEAST HEMOGLOBIN

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1. Introduction

Oxygen transport is considered to be the most important function of hemoglobin and myoglobin. Facilitated transport of oxygen may be an important function of myoglobin, and it has been suggested that the hemoglobin of microorganisms functions similarly [1]. Many reports concerning non-circulating hemoglobin have appeared in the literature [2–11]. Keilin originally discovered a hemoglobin-like pigment in yeast [2], but reported that over a period of 4 yr he had detected hemoglobin in bakers yeast only twice [2]. Because hemoglobin so seldom appears in yeast cells it has been difficult to make any extensive study of the properties of this hemoprotein. During our general survey of the hemoproteins in yeast, we have identified some species of *Candida* which consistently have a high hemoglobin content. We have subsequently succeeded in purifying this hemoglobin [12].

In this communication we will present measurements of the oxygen affinity of this hemoglobin as obtained using a sensitive bioluminescent oxygen indicator. The data provides evidence that the yeast hemoglobin binds oxygen non-cooperatively with high affinity and appears to be a "primitive" form of the hemoglobin of higher organisms. Moreover, the data indicates that yeast hemoglobin does not significantly enhance the diffusion of oxygen to the cytrochrome oxidase of the cells in which it is present.

2. Materials and methods

2.1. *Preparations*

A yeast, *Candida mycoderma*, which was obtained from the Institute for Fermentation, Osaka, Japan, was grown at 30° with aeration on a rotary-type shaker in polypeptone–yeast extract–glucose medium overnight.

The native cells contained hemoglobin and when hemoglobin-free cells were required they were prepared by suspension at 0° for 5 min in a 0.1 M phosphate buffer (pH 7.0) containing 0.8 mM ethylhydrogen peroxide (EtOOH) which decomposed the hemoglobin. The cells were then collected by centrifuging for 10 min at 5,000 *g* and resuspended in 0.1 M phosphate buffer pH 7.0. This EtOOH treatment did not affect the cellular respiration as measured by an oxygen electrode. The hemoglobin content in the EtOOH-pretreated cells, measured spectrophotometrically, was less than 10% of the original content. No alteration in the content of the other hemoproteins was observed.

Yeast hemoglobin was isolated from the supernatant fraction obtained when the cells were disrupted by a French press and the resulting suspension was centrifuged at 104,000 *g* for 60 min. Further purification was performed by ammonium sulfate fractionation, protamine treatment and chromatography on a diethylaminoethyl cellulose column. Details will be described elsewhere [12]. After dialyzing the supernatant fraction of 70% saturation with ammonium sulfate against water, this fraction was used as a methemoglobin reductase.

Sperm whale myoglobin was purchased from Sigma Chemical Co., *Saccharomyces cerevisiae* from the Universal Food Corporation and EtOOH from Ferrosan, Malmo, Sweden.

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2.2. *Physical techniques*

The oxygen equilibrium was measured essentially as described by Schindler [13]; the bioluminescence of *Photobacterium fisheri* (by courtesy of Dr. F.H. Johnson, Princeton University) was used as a sensitive oxygen indicator and absorbance measurements were used to determine the binding of oxygen to the hemoglobin. The reaction cuvette used for the measurement was provided with a gas inlet and outlet with a flowmeter. The light path was 5 cm. The reaction mixture had a total volume of 120 ml and the suspending medium containing 3% NaCl, 0.1 M phosphate buffer pH 7.0, 10 mM glucose and a trace of catalase to prevent H_2O_2 accumulation. The oxygen indicator *P. fisheri* (500–100 mg wet wt) and yeast cells (0.2–0.4 g wet wt), or yeast hemoglobin (10^{-7}–10^{-5} M) or sperm whale myoglobin (10^{-6} M) were then added to complete the reaction mixture. When isolated hemoglobin was used, the methemoglobin reductase was also added. The oxygen binding by hemoglobin and myoglobin was measured at 436 nm with respect to 410 nm using a Johnson Foundation dual wavelength spectrophotometer. Simultaneous measurements of the bioluminescence were achieved using an additional photomultiplier which was appropriately filtered to accept light of wavelengths greater than 450 nm. The bacterial luminescence was calibrated by addition of aliquots of oxygen-saturated water in the range 10^{-9}–10^{-5} M.

3. Results

The bioluminescence reaction of *P. fisheri* is oxygen dependent and has been used as a sensitive indicator for oxygen [13–16]. A typical experiment is shown in fig. 1. Argon gas is allowed to flow over the reaction mixture in a closed vessel in which the mixture is constantly stirred by a magnetic stirrer. Both the bacteria and yeast in the vessel consume oxygen and bring about a slow decrease in oxygen concentration. Luminescence starts to decrease when the oxygen concentration falls below 10^{-5} M. Simultaneous measurement of the change in absorbancy at a given wavelength, therefore, makes it possible to determine the effects of oxygen concentration on the oxido-reduction or deoxy-oxygenation reactions of the various biopigments as seen in fig. 1. The introduction

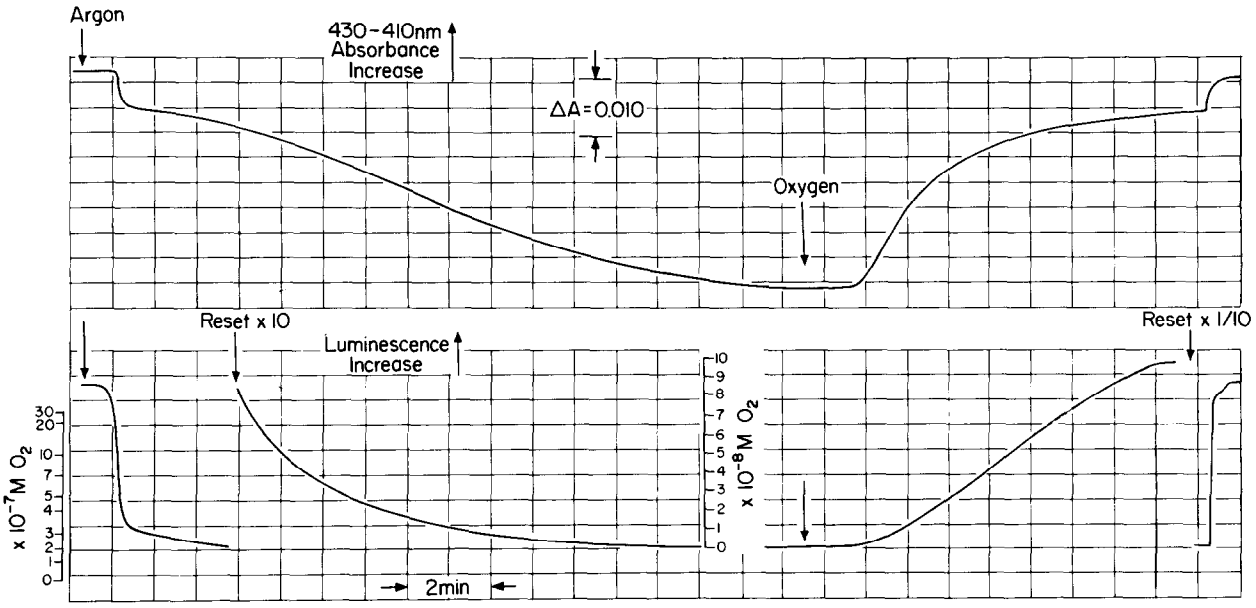

Fig. 1. Simultaneous recordings of luminescence and absorbance changes of hemoglobin in yeast cells. The reaction mixture contained (in a final volume of 120 ml) *C. mycoderma* (0.2 g wet wt), *P. fischeri* (0.05 g wet wt), 3% NaCl, 10 mM glucose, 0.1 M phosphate buffer pH 7.0 and a trace amount of catalase. The assay procedure was as described in Materials and methods.

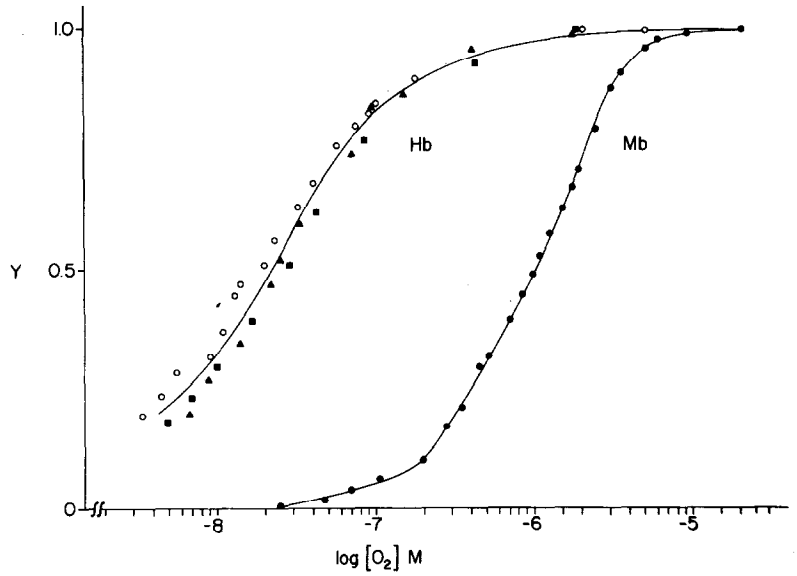

Fig. 2. Oxygen dissociation curves of isolated yeast hemoglobin and sperm whale myoglobin. The concentrations of yeast hemoglobin and sperm whale myoglobin used were 2×10^{-7} M and 1×10^{-6} M, respectively. The ordinate is expressed in terms of the fractional oxygen saturation ($Y = HbO_2/Hb + HbO_2$ or $MbO_2/Mb + MbO_2$). The measurements were performed at pH 6.0 (■), 7.0 (○) and 7.5 (▲) for yeast hemoglobin and at pH 7.0 (●) for sperm whale myoglobin. Other conditions were as described in Materials and methods.

of oxygen into the gas flow at the completely anaerobic state produces a slow recovery of both luminescence and absorbancy toward the original aerobic level, as the oxygen concentration in the mixture is increased. The change in absorbancy with respect to oxygen concentration remains exactly the same whether the data are taken during the deoxygenation or reoxygenation, thus supporting the validity of the assay method (cf. also fig. 3). Stepwise titration by increasing the oxygen concentration in the gas flow produces the same result.

The deoxygenation of isolated yeast hemoglobin and sperm whale myoglobin has been measured using this method. As shown in fig. 2, half oxygenation of sperm whale myoglobin was observed at an oxygen concentration of 10^{-6} M, corresponding to 0.67 mm Hg P_{O_2}. This value is in agreement with the value, 0.6 mm Hg at 20°, reported by Imai et al. [17]. Yeast hemoglobin has an affinity for oxygen about 100 times higher than does myoglobin; at Y = 0.5, the oxygen concentration is 2×10^{-8} M. The *n* value of the Hill equation was approximately 1 for both myoglobin and yeast hemoglobin and no significant Bohr effect in yeast hemoglobin could be detected in the pH range from 6.0 to 7.5 as shown in fig. 2.

Fig. 3 shows the oxygen concentration effect on yeast hemoglobin in the cells. Curve A represents the change in absorbancy at 436–410 nm by yeast hemoglobin, with some contribution to the absorbancy due to cytochromes. Curve B represents the residual cytochrome absorbancy change after disruption of hemoglobin with EtOOH treatment. By subtraction of curve B from curve A one obtains the absorbancy change due solely to yeast hemoglobin.

Experiments with *S. cerevisiae* gave curves similar to curve B regardless of whether the cells had been pretreated with EtOOH or not. This indicates that the strain of *S. cerevisiae* studied had no hemoglobin and that the absorbancy was due only to the cytochromes and, more important, that EtOOH pretreatment had no significant effect on cytochrome absorbancy. Curve A and B of fig. 3 may be used to estimate the hemoglobin oxygenation curve and a half-oxygenation value of 1.5×10^{-8} M is obtained. When the reaction mixture contained KCN (1 mM) the cytochromes were reduced but hemoglobin was not affected; the same oxygen affinity is found as shown in fig. 4, at Y = 0.5, the oxygen concentration is 2.0×10^{-8} M. This result has been confirmed by using different wave-

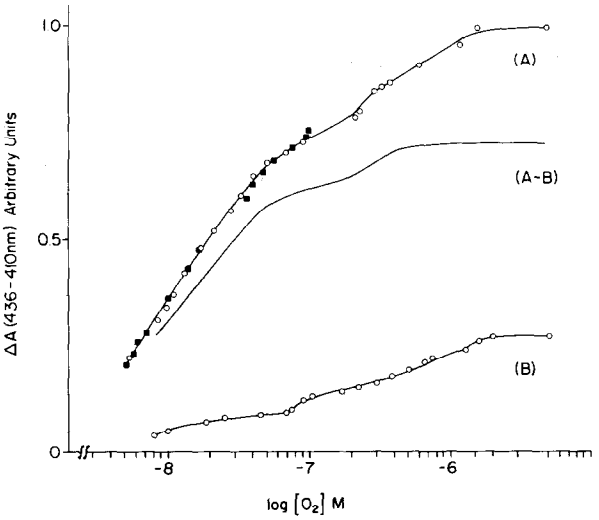

Fig. 3. Oxygen equilibrium of hemoglobin in yeast cells. The reaction conditions were as described in fig. 1. Curve A; normal cells. Curve B; EtOOH treated cells. The points ▪ and ○ represent the results calculated from the traces in aerobic to anaerobic and anaerobic to aerobic transitions, respectively. Trace A–B is obtained by subtracting the smoothed curves.

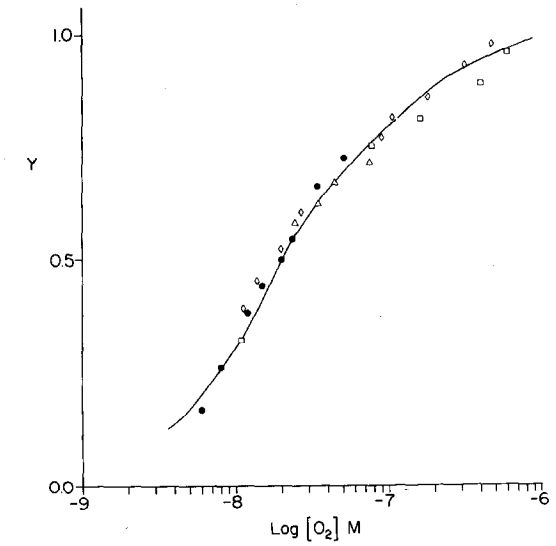

Fig. 4. Oxygen equilibrium of yeast hemoglobin in the cells treated by cyanide. The reaction conditions were as described for fig. 1 except that the concentration of bacteria was 0.1 g wet wt per 120 ml, and 1 mM KCN was added. Four different symbols represent the results from 4 different experiments.

lengths pairs (436–450 nm and 577–593 nm). In comparison with the results in fig. 3, these results emphasize that in spite of inhibition of cellular respiration by cyanide the oxygenation curve of yeast hemoglobin is the same in both systems within the experimental error.

4. Discussion

The oxygen affinity of the yeast hemoglobin is approximately 2×10^{-8} M (0.01 mg Hg) for both the isolated hemoprotein and the hemoprotein in the intact yeast cells. This value may be compared with the values for other non-circulation hemoglobins such as that of *Paramecium* (0.6 mm Hg, 9.3×10^{-7} M) [8], *Ascaris* body wall (0.1 mm Hg, 1.5×10^{-7} M) [18] and leg-hemoglobin (0.05 mm Hg, 7.8×10^{-8} M) [9]. The yeast hemoglobin has the highest affinity among these hemoglobins.

In order to test whether yeast hemoglobin can function to facilitate oxygen transport as had been postulated for myoglobin [19, 20], we have compared the K_m of oxygen for respiration in yeast cells which contain hemoglobin in the natural state with the K_m of oxygen for respiration of cells in which the hemoglobin had been decomposed by EtOOH treatment. No difference could be detected within the experimental error; the oxygen concentration giving a half maximal spectral change in cytochrome a_3 was about 3×10^{-8} M in both cases. This result suggests that the yeast hemoglobin is not functioning significantly to facilitate the diffusion of oxygen within the cells. This is a surprising result since the yeast hemoglobin has appropriate kinetic and equilibrium properties for facilitation of oxygen diffusion. First, its oxygen affinity is sufficient to maintain bound oxygen even at the low concentrations at which cytochrome oxidase can operate in these cells. Secondly, the dissociation velocity of oxygen ($k_{-1} = 17\ \text{sec}^{-1}$) is sufficiently rapid to meet the needs of cellular oxygen utilization (unpublished observations).

In summary, yeast serves as a most interesting and useful material for the investigation not only of oxidative metabolism [21–23], but also of the physiological relationship between hemoglobin and cytochrome oxidase. One might speculate that hemoglobin under primitive conditions suited the oxygen dissociation

curve for the cytochrome oxidase in single cells but as tissue diffusion gradients were increased and hemoglobin served an extracellular function, its oxygen affinity curve was progressively shifted toward a lower affinity which better served the needs for tissue oxygenation under conditions of a high diffusion gradient.

It further seems that yeast hemoglobin may be useful in studies on the structure and function of hemoglobin. On the one hand it is a primitive molecule without detectable cooperativity and on the other hand yeast metabolism and growth can be controlled so as to permit many modifications or substitutions in the molecule.

Acknowledgement

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