Yeast Flavohemoglobin from Candida norvegensis
ITS STRUCTURAL, SPECTRAL, AND STABILITY PROPERTIES*

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and Keiji Shikama

Flavohemoglobin was isolated directly from the yeast Candida norvegensis and studied on its structural, spectral, and stability properties. In Candida flavohemoglobin, the 155 N-terminal residues make a heme-containing domain, while the remaining 234 C-terminal residues serve as a FAD-containing reductase domain. A pair of His-95 and Gln-63 was assigned to the proximal and distal residues, respectively. In a purification procedure FAD was partially dissociated on a Butyl-Toyopearl column, so that FAD-lacking flavohemoglobin was also obtainable. In this ferric species, the Soret and charge-transfer bands were all characteristic of a pentacoordinate form. Compared with the recombinant heme domain expressed in Escherichia coli, we have measured the autoxidation rate over a wide pH range. The resulting pH dependence curve was then analyzed in terms of a nucleophilic displacement mechanism. As a result, the heme domain was found to be extremely susceptible to autoxidation, its rate being more than 100 times higher than that of sperm whale MbO₂. However, this inherently high oxidation rate was dramatically suppressed in Candida flavohemoglobin to an extent almost comparable to the stability of mammalian myoglobin. These new findings lead us to conclude that Candida flavohemoglobin, differently from bacterial flavohemoglobins, can serve as an oxygen storage protein in aerobic conditions.

In studies of nonvertebrate myoglobins and hemoglobins, we have disclosed, for the first time, the very unique structures of a protozoan myoglobin from Paramecium caudatum (1) and a yeast flavohemoglobin from Candida norvegensis (2, 3). Two such different types of globins are now established to occur widely in single-celled organisms. A first protein class, referred to as truncated hemoglobins, includes small hemoproteins characterized so far in the ciliated protozoa such as Paramecium caudatum, Tetrahymena pyriformis, and Thermococcus thermophila (1, 4, 5), in the unicellular green algae Chlamydomonas eugametos (6), and in the eubacteria such as Nostoc commune (7) and Mycobacterium tuberculosis (8). The present state of this topic has been reviewed recently by Wittenberg et al. (9). The second protein group, occurring in bacteria, yeast, and fungi, includes single-chain flavohemoglobins that consist of a N-terminal, heme-containing oxygen binding domain and a C-terminal, FAD-containing domain structurally related to NADH or NADPH reductase (10–15). Moreover, analysis of the microbial genomic sequences indicates that certain eubacteria, such as Bacillus subtilis and Staphylococcus aureus, contain both flavohemoglobin and truncated hemoglobin (16).

As a matter of course, various biochemical functions other than the conventional oxygen transport or storage have been proposed so far for these ancient hemoproteins, but the precise in vivo activity is still unclear. However, recent studies strongly suggest that the most probable role of bacterial flavohemoglobins is in the nitric oxide (NO) detoxification. In bacteria, NO is a very toxic agent that can be produced by the metabolic reduction of inorganic nitrogen oxides. For the Escherichia coli flavohemoglobin (Hmp), Gardner et al. (17) reported the aerobic conversion of NO to nitrate (NO₃⁻). Under anoxic reducing conditions, this protein was found to bind NO reversibly and to reduce it to NO₂⁻, which resulted in the formation of nitrous oxide (N₂O) via a dimeric reaction (18). These defenses against the nitrosative stress would be analogous to the well characterized antioxidant systems that protect aerobic organisms from reactive oxygen species.

In baker's yeast, Saccharomyces cerevisiae, flavohemoglobin is not always observable, its appearance being dependent largely upon the culture conditions. In another yeast cell, Candida norvegensis, on the other hand, Oshino et al. (13) found that flavohemoglobin occurred stably in a large enough amount for isolation and characterization. Taking this advantage, we have studied in the present paper the structural, spectral, and stability properties of Candida flavohemoglobin with special focus on the heme iron oxidation. Whatever the possible role of yeast flavohemoglobin may be (or might have been) in, including O₂-buffer, O₂-sensing, O₂⁻-production, NO-dioxigenation and oxidative stress response, the reversible binding of molecular oxygen to iron(II) must be the primary event to manifest their physiological functions in vivo.

MATERIALS AND METHODS

Materials—Butyl-Toyopearl (650 M) was a product of Tosoh Corp. (Tokyo, Japan). CM-cellulose (CM-32) and DEAE-cellulose (DE-32) were purchased from Whatman. Phenylmethylsulfonyl fluoride, FAD, and NADH were products of Sigma. Mes, Mops, Taps, Caps, and Tris for buffer systems and all other chemicals were of reagent grade from Wako.

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1 The abbreviations used are: NO, nitric oxide; Mes, Mops, Taps, Caps, and Tris for buffer systems and all other chemicals were of reagent grade from Wako.
Pure Chemicals (Osaka, Japan). Solutions were made with deionized and glass-distilled water.

Cell Culture—The seed cells of Candida norvegensis (IFO-0794) were cultivated for 30 h at 30 °C with constant shaking in a medium of 1.0% (w/v) glucose, 0.7% (w/v) peptone, 0.5% yeast extract, 0.05% KH₂PO₄, 0.05% MgCl₂, and 0.05% CaCl₂. The seed cells of 200 g were harvested from the mass culture of 23 liters, resuspended in an equal volume of 0.1 M Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, and kept at −80 °C until use.

Preparation of Candida Oxyhemoglobin—The frozen cell suspension (150 g in each experiment) was thawed quickly and digested with lysozyme (10 mg per g cells at 30 °C for 6 h) and the presence of 1 mM phenylmethylsulfonyl fluoride as proteinase inhibitor. Lysozyme 20T (Arthrobacter luteus) was purchased from Seikagaku Corp. (Tokyo, Japan). The cells were finally ruptured by sonication (Sonifier, model W-185, Tokyo, Japan) for 6 min at 150 W. After the lyzed cells were centrifuged down, the supernatant was fractionated with ammonium sulfate between 45 and 75% saturation. The reddish precipitate was then applied to a Butyl-Toyopearl column (2.6 × 10 cm), which had been equilibrated with 1 M (NH₄)₂SO₄ in 50 mM Tris-HCl buffer, pH 7.5. After the column was washed with a large volume of the equilibrium solution, elution was made with 50 mM Tris-HCl buffer alone. The resultant hemoglobin solution was further purified by gel filtration on a Sephadex G-75 column (5 × 90 cm) equilibrated with Tris-HCl buffer (pH 7.5) containing 5% glycerol and 1 mM EDTA. The essential step was the chromatographic separation of the oxygenated form of yeast hemoglobin on a DEAE-cellulose column (1.7 × 10 cm), with a linear concentration gradient of Tris-HCl buffer from 5 to 60 mM at pH 7.5. All these procedures were carried out at low temperature (0–4 °C) as far as possible.

The concentration of Candida flavohemoglobin was determined after conversion into cyanomet-form using an absorption coefficient of 11.2 mM⁻¹ cm⁻¹ at 540 nm. This value was obtained on the basis of the pyridine hemochromogen method (19). For each Hb solution, the FAD content was also determined after deproteinized with trichloroacetic acid 5% solution containing 0.2 M appropriate buffer and 2 mM EDTA. The absorbance was measured at 450 nm. A standard curve was prepared by comparing the regression of Hb solution (20). All experiments were conducted at least in triplicate.

Spectroscopic Measurements—Absorption spectra were recorded in a Hitachi two-wavelength double-beam spectrophotometer (model 557, U-3210 or U-3300). Fluorescence measurements were carried out in a Hitachi fluorometer (model F-2000). Each spectrometer was equipped with a thermostatically controlled cuvette (± 0.1 °C) cell holder. Circular dichroism (CD) spectra were obtained in a Jasco spectropolarimeter (model J-720) equipped with a thermostatically controlled cell holder. In the far ultraviolet zone, recordings were made with 10 μM hemoglobin in a 1-mm cell and at the scale setting of 0.002 degree/cm on the chart recorder.

Oxygenation Rate Measurements—According to our standard procedure, the rate of autoxidation of oxyhemoglobin was measured in 0.1 M buffer at 25 °C over a wide range of pH 5–10 and in the presence of 1 mM EDTA. A 500-μl solution containing 0.2 M appropriate buffer and 2 mM EDTA was placed in a semimicro-cuvette and incubated in a water bath maintained at 25 (± 0.1) °C. The reaction was started by adding the equal volume of fresh HbO₂ solution (20 μM), and changes in the absorption spectrum over 250–700 nm were recorded on the same chart at measured intervals of time. For the final state of each run, the hemoglobin was completely converted to the ferric met-form by the addition of potassium ferricyanide. The buffers used were Mes, Mops, Taps, Caps, and Tris. The pH value of the reaction mixture was carefully checked before and after the run with a Hitachi-Horiba pH meter (model F-22).

Construction and Expression of the Recombinant Heme Domain of Candida Flavohemoglobin—In our construct, the sequence of 155 N-terminal residues of Candida flavohemoglobin was designed to be expressed as a fusion product with maltose binding protein (MBP). The cDNA encoding this heme domain was prepared with a XmaI 1 site at the 5’ end and a BamHI 1 site at the 3’ end. The gene was ligated into the corresponding restriction sites of pMAL-c2 (New England Biolabs, Inc.) to construct the expression plasmid, pMAL-C2/heme domain (7.2 kbp). The expression plasmid was transformed into E. coli TB-1 cells (Invitrogen), and the cells were grown for 5 h at 37 °C in LB medium containing 100 μg/ml ampicillin and 0.1 mM β-aminolevulinic acid. The induction was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 0.1 mM, and expression was continued for 10 h with constant shaking. Cells were harvested, washed with 15 mM Tris-
A Structural Prediction of Candida Flavohemoglobin

<table>
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<th>1</th>
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<th>95</th>
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<td>Ser</td>
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Fig. 2. A structural prediction of Candida flavohemoglobin with two distinct domains of different helical contents. The helical fraction ($f_{\text{h}}$) was estimated on the basis of CD measurements shown in Fig. 3.

As depicted in Fig. 2, the N-terminal domain is the heme-containing region that binds molecular oxygen, whereas the C-terminal domain may serve as a FAD-containing reductase to convert the former from its ferric met-form to the ferrous deoxy-form in the presence of NADH.

In relevance to these structural prediction, we have carried out circular dichroism measurements for this protein in 10 mM phosphate buffer, pH 7.2. As a result, the mean residue molar ellipticity at 222 nm was found to be $[\theta]_{	ext{MRW}} = -15,000$ degree cm$^2$ dmol$^{-1}$. When compared with a value of $-25,400$ degree cm$^2$ dmol$^{-1}$ for sperm whale myoglobin, this CD magnitude was surprisingly low, and the total helical content was estimated to be 44% or less. Therefore, it seemed of great interest to test whether the heme-containing domain carry a common globin-fold or not. Using plasmid vector pMAL-c2, we have therefore prepared an expression system for the oxygen-binding domain has a CD magnitude of 24,000 degree cm$^2$ dmol$^{-1}$, this being almost identical to that of sperm whale myoglobin.

Consequently, the secondary structure of Candida flavohemoglobin may be depicted as follows. In the heme domain, approximately 70% of the residues can exist in a helical conformation. In Fig. 2, His-95 is assigned to the heme-binding proximal (F8) histidine, while Gln-63 is placed at the distal (E7) position. As for the remaining FAD-containing domain, its helical content was calculated to be 27% or less. Rather, this region is likely abundant in $\beta$-barrel and $\beta$-sheet structures, just like that of the bacterial flavohemoglobin from Alcaligenes eutrophus whose two-domain structure was already determined at 1.75 Å resolution by Ermler et al. (12). In the Candida protein, the probable FAD-binding sites were deduced to be Arg-213, Tyr-215, Ser-216, and Ser-237 based on the sequence comparison with other flavoproteins (11, 12, 24, 25). It should be added here that both in Saccharomyces and Alcaligenes flavohemoglobins, a different pair of Gln-53 and His-85 is aligned to the distal (E7) and proximal (F8) residues, respectively (12). The sequence homology between Alcaligenes and Candida flavohemoglobins was 28% overall.

In view of these structural features, yeast hemoglobin is very likely to have been derived from the fusion of two genes, one encoding a globin that binds a heme, and the other encoding a flavoprotein that has a reductase activity. Nevertheless, our comparison of the cDNA and genomic DNA sequences clearly indicated that Candida flavohemoglobin contains no introns in it. This result is in good accordance with that of Saccharomyces flavohemoglobin by Zhu and Riggs (11).

Isolation and Spectral Characterization of Candida HbO$_2$—

In relation to the structural evidence, our next step was to clarify the spectral and stability properties of Candida flavohemoglobin. We have therefore isolated native oxyhemoglobin directly from the yeast cells. After subjected to gel filtration on a Sephadex G-75 column, the hemoglobin solution (approximately 200 ml) was applied to a DEAE-cellulose column (1.7 × 10 cm) equilibrated with 5 mM Tris-HCl buffer, pH 7.5. The column was then washed with a large volume of the same buffer to remove contaminant proteins and also a small amount of the ferric methemoglobin (metHb) as well. To obtain the oxygennated hemoglobin (HbO$_2$), elution was made with a linear concentration gradient of Tris-HCl buffer from 5 to 60 mM at pH 7.5. Fig. 4 is such a typical elution profile to show that Candida oxyhemoglobin was usually obtained in two peaks. The first, small peak contained the FAD-lacking fraction with values of $f_{\text{FAD}} < 0.1$, while the second, major peak was for the FAD-bound fraction having values of $f_{\text{FAD}} > 0.8$. In this way, the yield of Candida flavohemoglobin amounted to 25 mg, including FAD-lacking species, from 150 g of the cells.

Fig. 5 represents the absorption spectra of Candida flavohemoglobin both in the oxy-form (continuous line) and in the acidic met-form (broken line). In the oxygenated flavohemoglobin, the $\alpha$-peak was lower than the $\beta$-peak with an absorbance ratio of $\alpha/\beta = 0.76$, this being in contrast to sperm whale MbO$_2$ having a value of 1.07. At the same time, a characteristic shoulder appeared at 480 nm, indicative of binding of an oxidized FAD group. The ultraviolet absorption peak also contains a large contribution from the bound FAD. In fact, this sample had a value of $f_{\text{FAD}} = 1.0$. The corresponding met-form was produced from HbO$_2$ solution by the addition of potassium ferricyanide. The residual oxidant was removed completely by
passing through a Sephadex G-25 column. The spectrophotometric parameters of Candida flavohemoglobin are summarized in Table I.

As a reference, the absorption spectra of the FAD-lacking hemoglobin (FAD-/H) are presented in Fig. 6, both in the oxy-form and in the ferric met-form. Here, the phrase "FAD-lacking hemoglobin" or simply "FAD(-)" denotes the intact whole protein but lacking only the FAD cofactor. As a result, the oxygenated form is almost identical to that of the FAD-bound oxyhemoglobin, while the ferric met-form was different from that of the FAD-bound protein. In particular, the Soret peak was profoundly blue-shifted and accompanied by a marked decrease in intensity, probably due to a broadening of the spectrum. In this respect, we have already proposed that myoglobins (or hemoglobins) can be divided into two groups on the basis of the absorbance ratio of the Soret peak of the acidic met-form to that of the oxy-form, that is to say the met/oxy ratio. Values higher than 1.0 were obtained for the usual type of myoglobins, whereas those of less than 1.0 were seen for the ratio of the myoglobins lacking the distal histidine residue (26). For such different positioning of the Soret band, our explanation is as follows: In the acidic metmyoglobins, the sixth coordinate position of the ferric heme iron is usually occupied by a water molecule, which can be stabilized by a hydrogen bond to the distal (E7) histidine residue. In case of myoglobins lacking the E7 histidine, the sixth coordinate position becomes vacant by the concomitant removal of a water molecule (27). We have confirmed this explanation by the magnetic circular dichroism spectroscopy in terms of the hexa- or penta-coordinate structure for the ferric high-spin myoglobins (28).

In the FAD-lacking species of Candida flavohemoglobin, a value of met/oxy = 0.71 was observed together with the appearance of a well-developed CT 2 band having maximum intensity of 9.6 M⁻¹ cm⁻¹ at 500 nm. The intensity of the CT 1 band was lowered to 2.7 M⁻¹ cm⁻¹ at 639 nm. All these spectral features are characteristic of a penta-coordinate form of ferric high-spin species and are in full accord with our assignment of Gln-63 to the distal E7 position. It follows from these facts that large changes in the heme environment result
from loss of only the FAD group. In the native flavohemoglobin (Fig. 5), its high value of $\gamma_{\text{met}}/\gamma_{\text{oxy}} = 1.18$ was also found to decrease markedly to 0.93 when the temperature was raised up to 40 °C. This indicates that the vacant-type species could be produced from the aqua met-form by thermal dissociation of the axial water molecule, as is in the case of African elephant myoglobin having Gln-64 as the distal residue (29). Incidentally, the crystal structure of Alcaligenes flavohemoglobin revealed that the sixth coordinate position is vacant in the ferric met-form (12, 30).

In preparation of Candida flavohemoglobin, it is a matter of our experience that the metHb content was usually maintained at a very low level, probably by a strong reduction with NADH present in the cells. As for the origin of the FAD-lacking species, it is not due to a shortage of FAD in the cells. Instead, a large quantity of FAD was released when the hemoglobin extract was subjected to a Butyl-Toyopearl column. We thus concluded that dissociation of the FAD group occurred from Candida flavohemoglobin during the course of such a hydrophobic interaction chromatography on Butyl-Toyopearl.

**Stability Properties of Candida Flavohemoglobin and Its Heme Domain**—In Candida flavohemoglobin, a value of $P_{50}$ = 0.01 Torr was reported by Oshino et al. (14), but the stability property of the bound dioxygen is entirely unknown. This property seems to be of particular importance to consider the possible role of Candida flavohemoglobin on the basis of its unique structure. At any rate, it is in the ferrous form that myoglobin or hemoglobin can bind molecular oxygen reversibly. Under air-saturated conditions, however, the oxygenated form is known to be oxidized easily to the ferric met-form, with generation of the superoxide anion as shown in Reaction 1 where $k_{\text{obs}}$ represents the first-order rate constant observed at a given pH value (31). Therefore, the rate of the autoxidation is written as follows.

$$\frac{-d[HbO_2]}{dt} = k_{\text{obs}}[HbO_2]$$  \hspace{1cm} (Eq. 1)

This process was followed by a plot of experimental data as $-\ln([HbO_2]/[HbO_2])$ versus time $t$, where the ratio of HbO$_2$ concentration after time $t$ to that at time $t = 0$ can be monitored by the absorbance changes at $\alpha$-peak of the oxygenated species (576 nm in the case of Candida HbO$_2$).

Fig. 7 shows such first-order plots to compare the autoxidation rate of Candida oxyhemoglobin with those of sperm whale, human psoas, and Paramecium oxymyoglobin under the same condition in 0.1 M phosphate buffer, pH 7.2, at 25 °C and in the presence of 1 mM EDTA. From the slope of each straight line the observed first-order rate constant ($k_{\text{obs}}$) was thus determined as follows: 0.50 × 10$^{-2}$ h$^{-1}$ for sperm whale MbO$_2$, 0.83 × 10$^{-2}$ h$^{-1}$ for human psoas MbO$_2$, 0.31 × 10$^{-2}$ h$^{-1}$ for Paramecium MbO$_2$, 0.24 × 10$^{-1}$ h$^{-1}$ for Candida flavohemoglobin, and 0.26 h$^{-1}$ for the FAD-lacking flavohemoglobin. Again, the label FAD(+) is used for the FAD-bound, native flavohemoglobin, whereas the FAD(−) denotes the intact whole protein but lacking only the FAD group.

It is thus clear that Candida flavohemoglobin is oxidized easily to the ferric met-form, its rate being somewhat slower than that of the protozoan myoglobin from *P. caudatum* but several times faster than that of sperm whale myoglobin. It is also clear that a loss of only FAD cofactor makes Candida flavohemoglobin extremely susceptible to autoxidation with a half-life period of 2.6 h. This high oxidation rate was surprising if compared with a value of $t_{1/2}$ = 29 h in the FAD-bound, native protein. Incidentally, sperm whale MbO$_2$ was oxidized with a half-life period of 138 h. Since the iron(III) species thus produced cannot bind molecular oxygen, the first-order rate constant ($k_{\text{obs}}$) can be employed as a useful measure for the stability of the heme-bound dioxygen (32).

$$\text{Hb(II)(O}_2\text{)} \rightarrow \text{metHb(III)} + \text{O}_2$$  \hspace{1cm} (Reaction 1)

If the values of $k_{\text{obs}}$ are plotted against the pH values of the solution, a profile of the stability of oxymyoglobin (or oxymy-
globin) can be obtained. Fig. 8 shows two such profiles, for *Candida* flavohemoglobin and its FAD-lacking species. In the flavohemoglobin, the rate of autoxidation increased with increasing hydrogen ion concentration but much less so than in sperm whale MbO₂. In the latter protein, a value close to $n = -1$ was found for the slope of $\log(k_{\text{obs}})$ versus pH, as will be seen later in Fig. 10. In *Candida* flavohemoglobin, its slope was less than $n = -0.4$, exhibiting rather a rate-saturation behavior at the extremely low pH values. This is a strong indication that *Candida* flavohemoglobin does not contain any proton-catalyzed process, such as the one that can play a dominant role in the autoxidation reaction of most mammalian oxymyoglobins (or oxyhemoglobins) that have the usual distal (E7) histidine residue. In *Candida* flavohemoglobin, a minimum rate appeared at pH 7, and a further increase occurred at the higher pH values. At the basic extreme, its rate came up to a level of the FAD-lacking species. Such an abrupt rate increase seemed to result from concomitant loss of the FAD cofactor from the reductase domain. In fact, the pH at which half of the bound FAD was dissociated from *Candida* flavohemoglobin was 8.6 at 25 °C (33).

At this point, it seemed of great interest to compare the stability property between the heme domain of *Candida* flavohemoglobin and the corresponding sperm whale MbO₂ since both proteins are of a size in amino acid residues (155 and 153, respectively). When the recombinant heme domain was placed in air-saturated buffers, the oxygenated domain was oxidized very quickly to the ferric met-form. Fig. 9 shows such spectral changes with time in 0.1 M phosphate buffer, pH 7.2, at 25 °C and in the presence of 1 mM EDTA. The reaction started from the fully oxygenated form with an absorbance ratio of $d\beta = 0.79$, and the spectra evolved with a set of isosbestic points (at 468, 528, and 590 nm) to the final state of the run, which was identified as a typical penta-coordinate form of the ferric high-spin species. Its Soret peak was profoundly blue-shifted and accompanied by a marked decrease in intensity. A very strong CT² band also appeared with maximum centered at 505 nm. All these spectral features were similar to that of the FAD-lacking flavohemoglobin. From the absorbance changes monitored at 577 nm in Fig. 9, a value of $k_{\text{obs}} = 0.72 \text{ h}^{-1}$ was obtained with a half-life period of $t_{1/2} = 58 \text{ min}$.

In this way, we have established, for the first time, a complete pH value profile for the stability of the recombinant heme domain in 0.1 M buffer at 25 °C. As shown in Fig. 10, the heme domain of *Candida* flavohemoglobin was extremely susceptible to autoxidation over the wide range of pH 5–9. Moreover, its pH dependence was unusual as compared with that of sperm whale MbO₂ served as a reference. For example, the heme domain was oxidized at an almost constant rate over the pH range of 7–9, its rate being nearly 1000 times higher than that of sperm whale MbO₂ at pH 9.0. Another marked difference was also found at acidic pH values. In sperm whale MbO₂, the rate of autoxidation increases rapidly with increasing hydrogen ion concentration, exhibiting a value close to $n = -1$ for the slope of $\log(k_{\text{obs}})$ versus pH. This is an indication of the presence of a very strong acid-catalysis performed by the distal histidine residue (32, 34, 35). In the heme domain only, the rate also increases with lowering pH but much less so ($n = -0.4$) than in sperm whale MbO₂. Rather, the heme domain showed a rate-saturation behavior below pH 6, although the data points could not fully be obtained because of the protein denaturation.
The Properties of C. flavohemoglobin

In the autoxidation reaction, pH values can affect the rate in many different ways. Recent kinetic and thermodynamic studies of the stability of mammalian oxyhemoglobins have shown that the autoxidation reaction is not a simple, dissociative loss of O$_2$ from MbO$_2$ but is due to a nucleophilic displacement of O$_2$ from MbO$_2$ by a water molecule or a hydroxyl ion that can enter the heme pocket from the surrounding solvent. The iron(II) is thus converted to the ferric met-form (32). Based on such a nucleophilic displacement mechanism, the pH profile of the autoxidation rate of sperm whale MbO$_2$ has already been analyzed completely in terms of an “acid-catalyzed two-state model” (27, 32, 36). In this model, it is assumed that a dissociable group, XH with pK$_a$, is involved in the reaction. Consequently, there are two forms of the MbO$_2$, represented by A and B, at molar fractions of $\alpha$ and $\beta = (1 - \alpha)$, respectively, which are in equilibrium with each other but which differ in dissociation state for the group XH. These forms can be oxidized to metMb by displacement of O$_2$ from MbO$_2$ by an entering water molecule or hydroxyl ion.

For this reaction, the observed rate constant $k_{obs}$ in Equation 1 can therefore be reduced as shown.

$$k_{obs} = \left[ k_0^A[H_2O] + k_0^B[H_2O][\text{H}] \right][\text{H}]$$

(Eq. 2)

where

$$\alpha = \frac{[\text{H}]}{[\text{H}] + K_a}$$

(Eq. 3)

In the above equation, $k_0$ is the rate constant for the basal displacement by H$_2$O, $k_H$ is the rate constant for the proton-catalyzed displacement by H$_2$O, and $k_{OH}$ is the rate constant for the displacement by OH$^-$. By iterative least-squares procedures inserting various values for $K_a$, the adjustable parameter in Equation 3, the best fit to the experimental values of $k_{obs}$ was obtained as a function of pH (Fig. 10). In this way, the rate constants and the acid dissociation constant involved in the autoxidation reaction of sperm whale MbO$_2$ were established in 0.1 m buffer at 25 °C as cited in Table II. From these results, it became evident that the proton-catalyzed processes with the rate constants $k_0^A$ and $k_0^B$ promote most of the autoxidation reaction of sperm whale MbO$_2$ above the basal processes in water with the rate constants $k_0^A$ and $k_0^B$. In this proton catalysis, the distal E7 histidine (the dissociable group XH with pK$_a$ = 6.2), which forms a hydrogen bond to the bound dioxygen (37), appears to facilitate the effective movement of a catalytic proton from the solvent to the bound, polarized dioxygen via its imidazole ring by a proton-relay mechanism (32, 38, 39). Random and undirected access of a proton to the bound dioxygen cannot yield such an enzyme-like, catalytic effect on the acidic autoxidation of MbO$_2$ or HbO$_2$.

Along with this line of evidence, kinetic formulation has now been made for the separated heme domain of Candida flavohemoglobin. Judging from only the data points shown in Fig. 10, it may be unclear that the reaction exhibits a rate-saturation behavior at extremely low pH values. However, its pH profile could be fully described by the following “two-state equation,” which contains a single dissociation process (for the group XH with pK$_a$ = 5.8) but no proton-catalyzed process,

$$k_{obs} = \left[ k_0^A[H_2O] \right][\alpha] + \left[ k_0^B[H_2O][\text{H}] \right][\beta]$$

(Eq. 4)

where the molar fractions of $\alpha$ and $\beta = (1 - \alpha)$ can be given by Equation 3, too. The resulting rate constants and acid dissociation constant involved in the autoxidation of the separated heme domain are summarized in Table II. As for the dissociable group XH with pK$_a$ = 6.2, we suggest that the most probable candidate is a carboxyl group of the heme propionates, just like the case of Aplysia MbO$_2$ lacking the distal histidine residue. When the protoshere was esterified with methanol to block its propionic acid side-chains, Aplysia myoglobin has lost completely such a rate increase with pK$_a$ = 6.1 (40).

In essence, the heme domain, if separated from Candida flavohemoglobin, undergoes extremely rapid autoxidation. At pH 7.2, for instance, its rate gave a value of $k_{obs} = 0.72$ h$^{-1}$ with a half-life period ($t_{1/2}$) of less than 1 h. However, this inherently high oxidation rate was dramatically suppressed in Candida flavohemoglobin. At pH 7.2, the half-life period of...
The Properties of C. flavohemoglobin

**TABLE II**

<table>
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<th>Source</th>
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<th>$k_b$ (h$^{-1}$ m$^{-1}$)</th>
<th>$k_{OH}$ (h$^{-1}$ m$^{-1}$)</th>
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<td>0.37 × 10$^{3}$</td>
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<td>0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B (X$^-$)</td>
<td>0.11 × 10$^{-1}$</td>
<td></td>
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</tr>
</tbody>
</table>

Heme oxidation was prolonged by 2.6 h in the FAD-lacking species and by up to 29 h in the native flavohemoglobin. We thus conclude that the binding of FAD as well as the attachment of the reductase domain can produce extensive changes in the distal heme pocket of Candida hemoglobin so as to block entering water molecules from the FeO$_2$ center. At present, however, we do not know whether the noncovalent presence of the reductase domain would be sufficient or whether the covalent attachment to the heme domain is indispensable. Nevertheless, the stability of the oxygenated form of Candida flavohemoglobin is quite different from the bacterial flavohemoglobins.

**DISCUSSION**

Single-chain flavohemoproteins are known to occur widely in lower organisms such as bacteria, yeast, and fungi. Among those, bacterial flavohemoglobins have been most extensively studied. As a result, it is now widely accepted that they are unlikely to serve as oxygen storage proteins. Rather, increasing attention has been directed to the nitric oxide detoxification for other, oxygen-binding heme proteins (32). From known changes in valency of the heme iron, one can write the functional cycle of hemoglobin as shown in Reaction 2.

In yeast flavohemoglobin, the situation is different. Even if the protein was oxidized to the ferric met-form, it can easily be reduced back to the ferrous state again by the addition of NADH alone. Actually, the oxidation and reduction cycle of Candida flavohemoglobin was followed up by the absorbance changes at 576 nm ($\alpha$-peak) in 0.1 M buffer at 25 °C (33). At first, Candida flavohemoglobin was oxidized gradually to the ferric met-form. When the oxidation reached a 60% level, for example, five equivalents of NADH were added to the solution. The metHb formed was reduced and oxygenated instantaneously to the almost original level of HbO$_2$. This reaction cycle could be repeated several times until the globin suffered from denaturation. In the FAD-lacking flavohemoglobin, such an electron transfer from NADH to the ferric heme iron was not observed as a matter of course. In this sense, Candida hemoglobin is a molecular system carrying its own reductase within the same molecule.

In the present paper, we have revealed for the first time that the heme domain, if separated from Candida flavohemoglobin, becomes extremely susceptible to autoxidation over the whole range of pH values studied. At pH 7.2, for instance, its oxidation rate was more than 100 times higher than that of the corresponding sperm whale MbO$_2$. Consequently, this oxidation rate would be too fast for the protein to play a role in oxygen buffer or oxygen storage. However, a very unique structure of Candida flavohemoglobin tells us another strategy to overcome many difficulties in the reversible and stable binding of molecular oxygen as opposed to the irreversible oxidation of heme iron(II) in aqueous environment. In view of the correspondence of yeast and bacterial flavohemoglobins, Zhu and Riggs (11) remarked that the two-domain structure has been conserved intact for as long as 1.8 billion years, the estimated time of divergence of prokaryotes and eukaryotes.

At all rates, our kinetic results presented here clearly indicate that Candida yeast flavohemoglobin, differently from bacterial flavohemoglobins, can serve as an oxygen storage protein in aerobic condition. This conclusion does not always rule out additional functions since the autoxidation rates of Candida flavohemoglobin are much too slow to interfere with catalytic activities such as nitric-oxide dioxygenase activity.
Yeast Flavohemoglobin from *Candida norvegensis*: ITS STRUCTURAL, SPECTRAL, AND STABILITY PROPERTIES

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