Relationship between the Stability and Autoxidation of Myoglobin

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The rate of autoxidation and the free energy of guanidine hydrochloride denaturation of myoglobin from sperm whale, horse, mackerel, skipjack, and bluefin, yellowfin, and bigeye tunas were studied in the pH range from 4.5 to 10.0. Myoglobin from tunas showed minimal autoxidation rate at pH 7.0–8.0 and the highest free energy at pH 7.5–8.0. Higher rates of autoxidation and lower values of free energy were observed at lower or higher pH values. The myoglobin stability was in the order bluefin tuna > yellowfin tuna > bigeye tuna, and the autoxidation rates showed the contrary order: bigeye tuna > yellowfin tina > bluefin tuna. The pH dependency of myoglobin from skipjack, mackerel, horse, and sperm whale was similar. Lower myoglobin stability was associated with higher autoxidation rate. High correlation coefficients between the rate constant and the free energy were observed.

The red color is regarded as an important parameter of meat and dark fish quality. The discoloration of tuna from red to brown during ice storage is induced by autoxidation of oxymyoglobin (oxyMb) to metmyoglobin (metMb). The autoxidation of oxyMb proceeds as a first-order reaction with respect to the remaining oxyMb (George and Stratmann, 1952a,b) and is highly dependent on pH. The autoxidation rate constant (k) increases with decreases in pH in the range pH 5–7 (Matsuura et al., 1962; Brown and Mebine, 1969; Gotoh and Shikama, 1974; Shikama and Sugawara, 1978). At higher pH, the pH profile of the autoxidation rate constant of bovine heart Mb showed a minimum at pH 9 and increased considerably from pH 9 to 10. They also reported that the activation energy (E_a) of Mb autoxidation was shown to be almost constant over the pH range 5–10 (Gotoh and Shikama, 1974). However, the activation energy of Mb was different at pH 5–7 from that outside this pH range (Brown and Mebine, 1969). The results on pH effects on autoxidation rate and E_a seem to be in disagreement.

The transition temperature of reversible denaturation of sperm whale Mb dropped very rapidly as the pH increased from 9 to 13 (Acampora and Hermans, 1967). The free energy for unfolding (ΔG_D) of sperm whale or horse Mb by guanidine hydrochloride (Gdn-HCl) denaturation showed a pH dependence that decreased with decrease in pH from pH 6.5 to 4 and reached a plateau around pH 7 (Puett, 1973). Free energy required to denature a protein is one of the parameters to evaluate "stability" against chemical denaturant (Ahmad and Bigelow, 1982; Greene and Pace, 1974). The results reported indicate that the environmental pH may simultaneously affect the stability and the autoxidation of Mb regardless of its origin.

The purpose of this study was to understand the relationship between the autoxidation rate and the stability of Mb under different pH conditions by using free energy for unfolding as a parameter. The effect of pH on the autoxidation rate of Mb was also studied.

MATERIALS AND METHODS

Materials. Fresh bluefin tuna (*Thunnus thynnus*), yellowfin tuna (*T. albacares*), and bigeye tuna (*T. obesus*), ice-chilled for less than 1 week, were purchased from Tung-Kang fish market. Fresh skipjack (*Katsuwonus pelamis*) and mackerel (*Pneumatophorus tapeinocephalus*) stored in ice aboard ship for 1 day were bought from Sue-Au fish market. Dark muscle was taken from those samples used for preparation of Mb soon after purchasing. Horse and sperm whale Mb and Gdn-HCl were obtained from Sigma. All other chemicals used were of analytical grade.

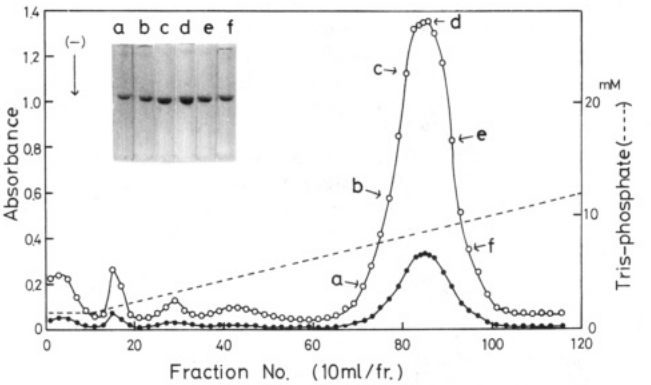

Figure 1. Chromatography of yellowfin tuna Mb on CM-Sephadex C-50. Mb was applied to a CM-Sephadex C-50 column (2.5 × 35 cm) equilibrated with 1.5 mM Tris–phosphate buffer (pH 7.0) and then was eluted by a linear gradient from 1.5 to 15 mM Tris–phosphate buffer (pH 7.0) at a flow rate of 60 mL/h. Protein was monitored at 280 (○) and 540 nm (●). The insert shows the SDS–PAGE patterns of the chromatographic fractions a–f.

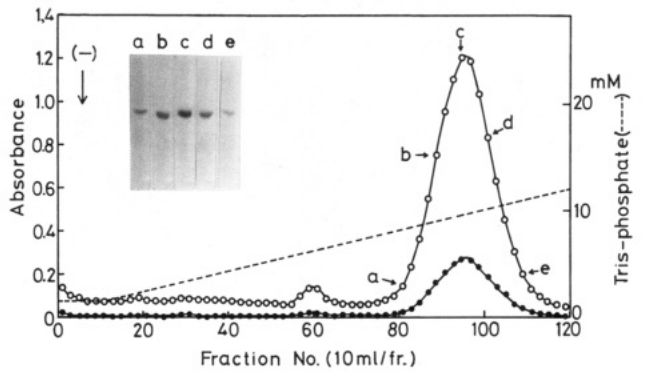

Figure 2. Chromatography of bigeye tuna Mb on CM-Sephadex C-50. The insert shows the SDS–PAGE patterns of chromatographic fractions a–e.

Myoglobin Preparation. Mb from yellowfin tuna and bigeye tuna Mb were prepared according to the method reported previously (Chow et al., 1985). Elution profiles of yellowfin tuna and bigeye tuna Mb from a CM-Sephadex C-50 column (2.5 × 35 cm) are shown in Figures 1 and 2. The homogeneity of the fractions was confirmed with sodium dodecyl sulfate–polyacryl-

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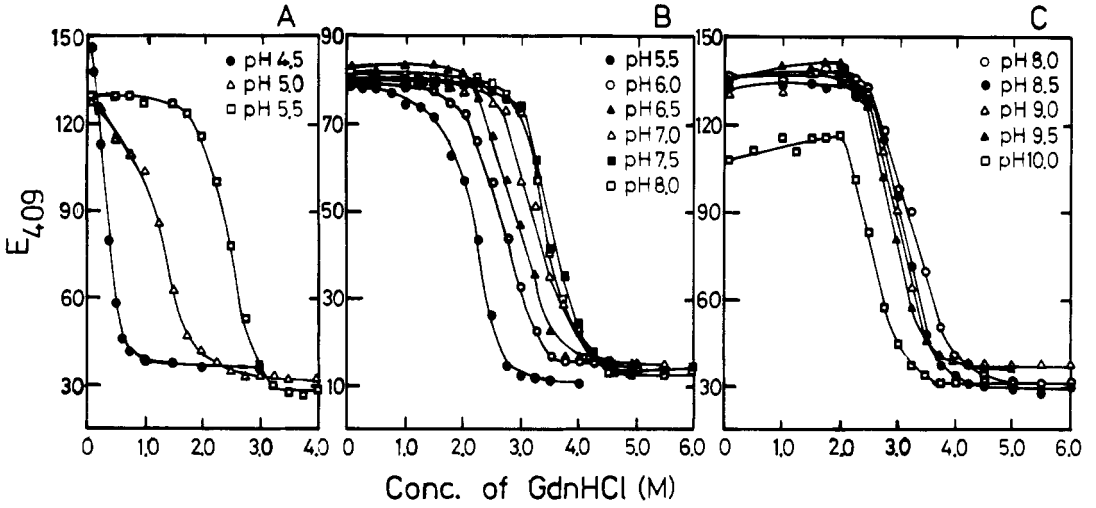

Figure 3. Changes in the Soret band (409 nm) of extinction coefficient of yellowfin tuna Mb on the concentration of Gdn-HCl at various pH values under equilibrium conditions. (A) 0.1 M sodium acetate buffer; (B) 50 mM sodium phosphate buffer; (C) 0.1 M glycine/NaOH buffer.

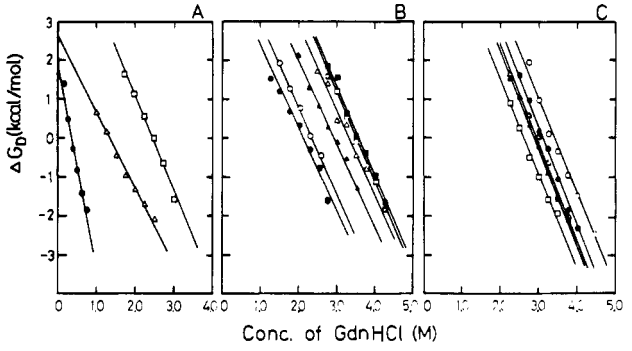

Figure 4. Influence of Gdn-HCl concentration on the free energy (ΔG_D) for unfolding the yellowfin tuna Mb. The lines are theoretically calculated from Figure 3 by using eqs 1 and 2 in the text. (A) 0.1 M sodium acetate (pH 4.5, ●; pH 5.0, △; pH 5.5, □); (B) 50 mM sodium phosphate buffer (pH 5.5, ●; pH 6.0, ○; pH 6.5, ▲; pH 7.0, △; pH 7.5, ■; pH 8.0, □); (C) 0.1 M glycine/NaOH buffer (pH 8.0, ○; pH 8.5, ●; pH 9.0, △; pH 9.5, ▲; pH 10.0, □).

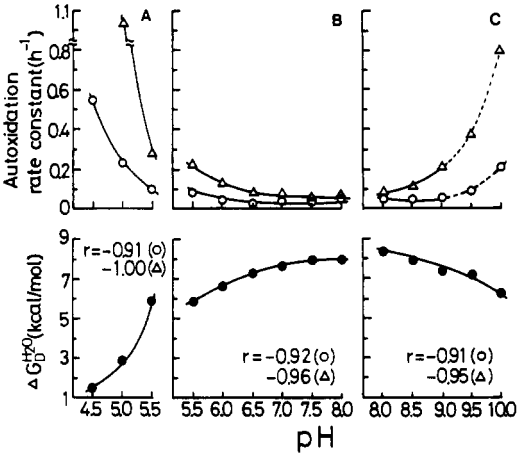

Figure 5. pH dependency of autoxidation rate constant (k) and free energy in the absence of Gdn-HCl ($\Delta G_D^{H_2O}$) of yellowfin tuna Mb. The autoxidation rate constant was measured at 20 (○) and 30 °C (△), and the free energy (●) was calculated by extrapolation from Figure 4 according to eq 3 in the text. The dotted line represents the estimation value of rate constant. (A) 0.1 M sodium acetate buffer; (B) 50 mM sodium phosphate buffer; (C) 0.1 M glycine/NaOH buffer. r represents the correlation coefficient between the free energy and the rate constant at 20 and 30 °C, respectively.

amide electrophoresis (SDS–PAGE) using 12.5% tube gels according to the method of Weber and Osborn (1969).

Skipjack and mackerel Mb were extracted from dark muscle with water. The fractionation between 80 and 90% saturation with ammonium sulfate at neutral pH was subjected to gel filtration. In the case of skipjack, a Sephadex G-75 column (5 × 85 cm) equilibriated with 50 mM Tris-HCl buffer (pH 8.0) was used, while a Sephadex G-50 column (3 × 90 cm) equilibrated with 0.1 M phosphate buffer (pH 6.5) was used for the purification of mackerel Mb.

Sperm whale and horse Mb were dissolved in a minimal volume of water and then applied to gel filtration by using Sephadex G-50 and Sephadex G-75 columns, respectively.

Purity of the main fraction from the columns was checked by disc SDS–PAGE, and only one band was observed in the patterns of skipjack, mackerel, horse, and sperm whale Mb (data not shown). The isolated Mb was immediately used in the following experiments. The Mb thus provided was of met form.

The concentration of Mb was determined by using a molar extinction coefficient of 11 300 for cyanometMb at 540 nm (Drabkin, 1945).

Measurement of Autoxidation Rate. Portions of the Mb solutions were dialyzed against 0.1 M sodium acetate buffer (pH 4.5–5.5), 0.05 M sodium phosphate buffer (pH 5.5–8.0), and 0.1 M glycine/NaOH buffer (pH 8.0–10.0), respectively. The final concentration of Mb was adjusted to 0.2–0.3 mg/mL. To each solution was added a trace amount of sodium hydrosulfite to convert the Mb into deoxyMb, which was then gently agitated to convert to oxyMb.

The autoxidation rate constant was determined according to the method of Matsuura et al. (1962) with some modifications as reported previously (Chow et al., 1985, 1987, 1989).

Determination of Free Energy for Unfolding. The free energy of Mb was measured by Gdn-HCl denaturation according to the method reported previously (Chow et al., 1989). Briefly, each dialyzed metMb solution was mixed with stock Gdn-HCl solution (both in the same pH), and the concentration of denaturant was 0.5–5 M after dilution. These solutions were incubated at 20 °C for a time long enough to complete the denaturation. Their Soret absorbances at 409 nm were recorded.

The equilibrium constant (K_D) in denaturation was calculated from the equation

$$K_D = \frac{f_D}{1-f_D} = \frac{E_N - E_{obs}}{E_{obs} - E_D} \quad (1)$$

where f_D is the fraction of denatured protein, E_N and E_D are the molar extinction coefficients of the protein in native and denatured states, respectively, and E_{obs} is the observed molar extinction coefficient of the protein transition between native and denatured states.

The free energy for unfolding or denaturation, ΔG_D, was determined from the experimental data by using the equation

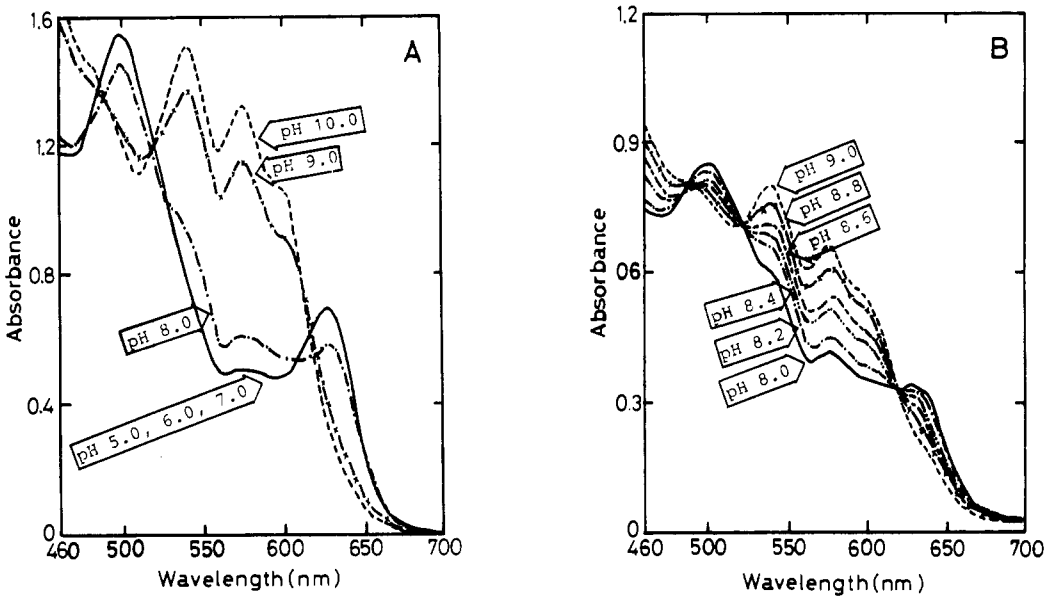

Figure 6. (A) Absorption spectra of yellowfin tuna metMb solutions under pH values from pH 5.0 to 10.0. The concentration of Mb was 0.64 mM. (B) Absorption spectra of yellowfin tuna metMb solutions under pH values from pH 8.0 to 9.0. The concentration of Mb was 0.21 mM.

$$\Delta G_D = -RT \ln K_D \quad (2)$$

where R is the gas constant [1.987 cal/(deg·mol)] and T the absolute temperature. The intrinsic value for ΔG_D of Mb was obtained by using the linear correlation between the value of ΔG_D and Gdn-HCl concentration. Therefore, the free energy stability of protein in the absence of denaturant ($\Delta G_D^{H_2O}$) was calculated by the equation

$$\Delta G_D^{H_2O} = \Delta G_D + mC \quad (3)$$

where C represents the molar concentration of the denaturant and m the slope of regression line.

All determinations were done in duplicate, and the mean values are reported.

RESULTS AND DISCUSSION

The Soret band of metMb at 409 nm results mainly from the interaction of the heme moiety with apomyoglobin and hence can be used to monitor the unfolding of hemoproteins (Acampora and Hermans, 1967; Puett, 1973). Changes in the Soret band of yellowfin tuna Mb as a function of Gdn-HCl concentration are shown in Figure 3. Higher concentrations of Gdn-HCl were required to unfold the Mb completely at neutral pH than at acidic pH (Figure 3A,B). As the pH was increased over 8, however, less denaturant was needed than at pH 8 (Figure 3C). The Mb in the sodium phosphate buffer showed a lower extinction coefficient than that in different buffer systems or pH conditions, but the reason remains to be elucidated.

The changes of free energy for Gdn-HCl denaturation (ΔG_D), calculated from the data in Figure 3 by using eqs 1 and 2, are shown in Figure 4. The ΔG_D values were dependent on pH and Gdn-HCl concentration for each Mb solution. Under the same concentration of denaturant, the ΔG_D values of Mb at acidic pH were smaller than at neutral pH (Figure 4A,B), while the contrary order was observed at pH values over 8 (Figure 4C).

The free energy stability of Mb in the absence of denaturant ($\Delta G_D^{H_2O}$) was calculated by linear extrapolation of the free energy of denaturation to zero denaturant concentration by using eq 3.

On the other hand, the autoxidation of yellowfin tuna oxyMb proceeded as the first-order reaction with respect to the remaining oxyMb, at each pH level and each buffer system (data not shown). The pH dependency of the autoxidation rate constant along with $\Delta G_D^{H_2O}$ of yellowfin tuna Mb is shown in Figure 5.

The autoxidation rate constant decreased with increases in pH from 4.5 to 7.0 and reached a plateau at pH between 7.0 and 8.0. However, the rate increased as the value of pH was increased over 8. The rate constant generally was higher at 30 °C than at 20 °C.

At pH above 9.0, the rate constant was difficult to measure since the absorption spectrum of metMb solution was similar to that of oxyMb solution. The former showed two absorption peaks at 540 and 575 nm as did the latter, which also showed a shoulder at 600 nm (Figure 6A). The metMb solutions at pH 8.0–9.0 showed a continuous change of spectra from metMb pattern to oxyMb pattern (Figure 6B). This spectral transition represents the change from the aquoferric derivative to the hydroxide ferric derivative. Therefore, the difference of absorbance between oxyMb and metMb at 568 nm was not accurately measurable for calculation of the metMb formation related to total Mb.

The value of $\Delta G_D^{H_2O}$ increased with increasing pH from 4.5 to 7.5 and remained almost constant (about 8 kcal/mol) at pH 7.5–8.0 (Figure 5A,B). However, $\Delta G_D^{H_2O}$ values decreased as the pH increased from 8.0 to 10.0. There showed a trend that the autoxidation rate and free energy of denaturation of Mb were inversely related, i.e., the lower the $\Delta G_D^{H_2O}$ value of Mb, the higher the autoxidation rate. the pH affected the former to a lesser extent than the latter. All correlation coefficients between the rate constant and the $\Delta G_D^{H_2O}$ value were considerably high at each buffer system and each incubation temperature.

Differences in autoxidation rate constant and $\Delta G_D^{H_2O}$ values of bluefin tuna and those of bigeye tuna Mb are shown in Figures 7 and 8. The patterns of both autoxidation rate and free energy of bluefin were similar to those of yellowfin tuna Mb. Both tuna Mb showed minimal rate constants at pH between 7.0 and 8.0. The $\Delta G_D^{H_2O}$ value of bluefin tuna Mb was maximal at pH around 8.0, while that of bigeye tuna Mb was between pH 7.5 and 8.0. High correlation coefficients were also observed.

The autoxidation rate constants of three kinds of tuna Mb were in the order bigeye tuna > yellowfin tuna > blue-

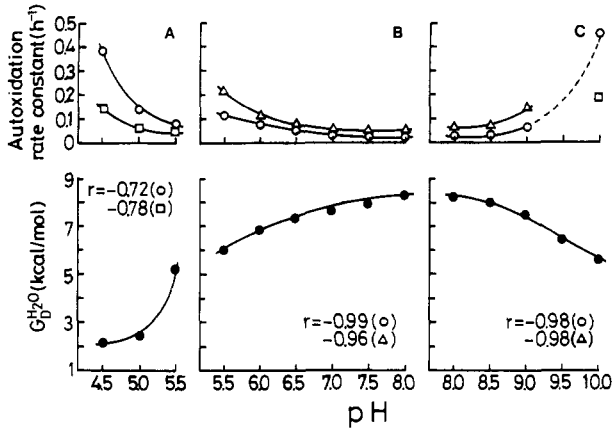

Figure 7. pH dependency of autoxidation rate constant (k) and $\Delta G_D^{H_2O}$ values of bluefin tuna Mb. The autoxidation rate constant was measured at 10 (□), 20 (○), and 30 °C (△), respectively. Refer to the legend of Figure 5.

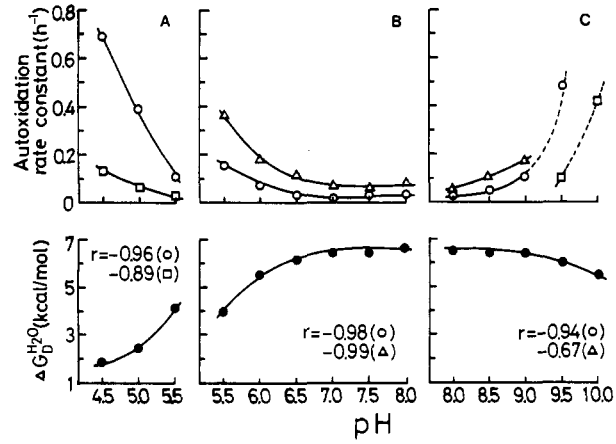

Figure 8. pH dependency of autoxidation rate constant (k) and $\Delta G_D^{H_2O}$ values of bigeye tuna Mb. The autoxidation rate constant was measured at 10 (□), 20 (○), and 30 °C (△), respectively. Refer to the legend of Figure 5.

fin tuna, regardless of incubation temperatures. The free energy for unfolding was in the order bigeye tuna < yellowfin tuna < bluefin tuna.

A similar relationship of autoxidation rate constants and $\Delta G_D^{H_2O}$ was found in skipjack and mackerel Mb (Figure 9). Both Mb showed a pH profile having a plateau in autoxidation rate or free energy between pH 7.5 and 8.0. Skipjack Mb had higher $\Delta G_D^{H_2O}$ values and lower autoxidation rate constants than did mackerel. Considering also the Mb from three different tunas, all showed a trend that higher $\Delta G_D^{H_2O}$ values were associated with lower rate constants. The correlation coefficients were high for both skipjack and mackerel Mb.

The pH dependency of the autoxidation rates and $\Delta G_D^{H_2O}$ of horse and sperm whale Mb is shown in Figure 10. Sperm whale Mb showed higher free energy and lower rate constants than did horse Mb. The pH profiles of Mb obtained from these two mammalia were very similar to those from fishes. The $\Delta G_D^{H_2O}$ values of sperm whale and horse Mb were quite close to those of tunas. However, the autoxidation rate constants of the former two were much less than those of the latter.

Matsuura et al. (1962) found that the autoxidation rates of horse and whale Mb were much slower than those of tuna and skipjack Mb. The present study was in agreement with their work. Shikama and Sugawara (1978) found that the rate constant of bovine Mb decreased pro-

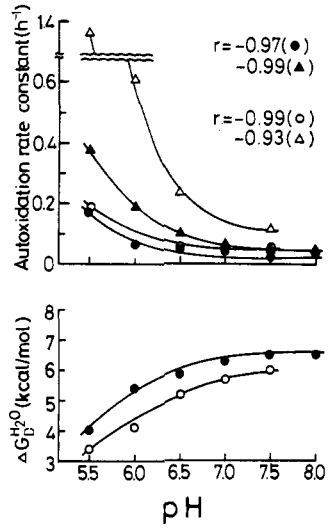

Figure 9. pH dependency of autoxidation rate constant (k) and $\Delta G_D^{H_2O}$ values of skipjack (solid symbols) and mackerel (open symbols) Mb. Refer to the legend of Figure 5.

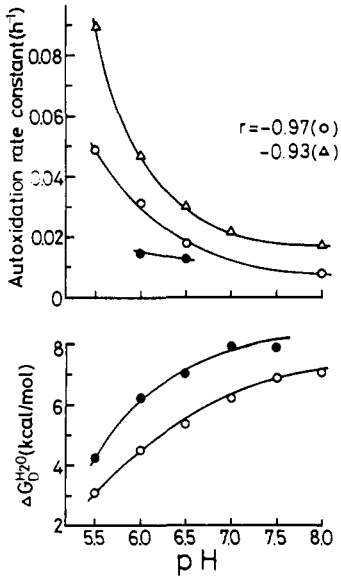

Figure 10. pH dependency of autoxidation rate constant and $\Delta G_D^{H_2O}$ values of horse (open symbols) and sperm whale (solid symbols) Mb. Refer to the legend of Figure 5.

nouncedly from pH 4.8 to 8.0 and showed a minimum at pH 9 and increased slightly as pH increased from 9 to 12.6. This study also showed similar pH dependence, except, the minimal rate constant was found between pH 7.0 and 8.0 for tuna Mb.

$\Delta G_D^{H_2O}$ values obtained in this study were smaller than those reported for sperm whale Mb (13.6 kcal/mol) (Puett, 1973) and horse Mb [11.0 kcal/mol (Puett, 1973), 10.1 kcal/mol (Pace and Vanderburg, 1979), or 7.9 kcal/mol (Ahmad and Bigelow, 1982)], respectively. These discrepancies may be attributed partly to different calculation methods adopted (Pace and Vanderburg, 1979) or different buffer conditions used (Chow et al., 1989).

Gdn-HCl is known as a strong denaturant, changing α-helix of protein into random-coil structure (Tanford, 1968; Puett, 1973). Since less denaturant was required when the pH was reduced from 7.5 to 4.5 or increased from 8.0 to 10.0 (Figures 5, 7, and 8), this indicated the α-helix portion of Mb was weakened at acidic or alkaline pH. Therefore, the Mb were able to unfold more easily. Furthermore, the weakened secondary structure may have

affected the stability of linkage between the heme and globin, where the oxygen molecule was loosely bound to the heme iron, causing the ferrous heme to be more susceptible to autoxidation.

In conclusion, there was a close relationship between the autoxidation rate of Mb and its stability against denaturation, especially among the Mb from dark-fleshed fishes: the higher the stability of Mb against denaturation, the lower the autoxidation rate constant.

ABBREVIATIONS USED

ΔG_D, free energy for unfolding of a protein; $\Delta G_D^{H_2O}$, free energy for unfolding a protein in the absence of denaturant; Mb, myoglobin; metMb, metmyoglobin; oxyMb, oxymyoglobin; Gdn-HCl, guanidine hydrochloride.

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