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Ionic Strength Effect on the Ascorbate Reduction of Sperm Whale and Horse Heart Metmyoglobins

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Abstract

Kinetics of the reduction of sperm whale skeletal muscle and horse heart metmyoglobins (metMb) by ascorbic acid (H_2A) were studied in a nitrogen atmosphere at 25 °C, at different ionic strengths (NaCl) and between pH 7.29 and 8.29. The second-order rate constants were determined for the reductions of metMb(H_2O) by ascorbate (HA^- and A^{2-}). It was found that the ascorbate reduction is faster for horse heart metMb than for sperm whale metMb at a higher ionic strength, and that at a lower ionic strength similar reactivities are observed. The logarithm of the rate constants correlates to the square root of the ionic strength. This is explained by a simple outer-sphere electron transfer mechanism. It was found that the effective charge of sperm whale metMb is higher than that of horse heart metMb and that there is no specific interaction of ascorbate with metMb.

Introduction

Redox kinetics of myoglobins (Mb) have been studied extensively using inorganic reagents, dithionite [1–5] and metal complexes [6–9]. Few kinetics, however, have been reported on the reduction of metMb with organic reductants [10, 11]. In a previous paper we reported the ascorbate reduction of sperm whale skeletal muscle metMb, in which the prosthetic group is protohemin [11]. It is suggested that the expansion of the heme pocket accelerates the ascorbate reduction of metMb. Horse heart metMb has the same prosthetic group as sperm whale metMb and both are similar in the heme environment, but there are 20 amino acid differences [12].

The difference in O_2 affinity of both myoglobins is small, but apparently sperm whale Mb has a higher affinity than horse heart Mb [13]. The urea or guanidine hydrochloride denaturation studies of metMb under equilibrium conditions show that sperm whale metMb is more stable than horse heart metMb due to conformation differences in localized regions; sperm whale metMb has a net of two additional Arg–Asp side chain interactions that cannot be found in horse heart metMb [14, 15].

It is interesting to study the effect of the structural perturbations in globin on the rate of reductions of metMb. In this paper we report the kinetics of the ascorbate reductions of sperm whale and horse heart metMbs at different ionic strengths and will discuss the reactivity differences of both Mbs.

Experimental

Materials

Sperm whale skeletal muscle myoglobin (Sigma, type II) and horse heart myoglobin (Sigma, type III) were purified by a CM-cellulose column chromatography. The sample was dissolved in a 5×10^{-3} M potassium phosphate buffer (pH 6.0) and was oxidized by $K_3[Fe(CN)_6]$. The solution was poured on a Whatman CM-52 cellulose column which had previously been equilibrated with a 5×10^{-3} M potassium phosphate buffer (pH 6.0). After the column was washed with the same buffer solution, the main fraction of metMb (middle of three fractions for sperm whale and the upper of two fractions for horse heart) was eluted with a 5×10^{-2} M potassium phosphate buffer (pH 7.2). Some remained on the top of the column. Horse heart metMb was more easily eluted than sperm whale metMb on a CM-cellulose column under the above conditions. The eluate was then dialysed against deionized water several times. All procedures were carried out at 4 °C. Horse heart metMb was found to be more easily denatured than sperm whale metMb when the solutions were stored in a refrigerator for a few months. The solutions of metMb for measurements were discarded after 3 weeks.

The concentrations of metMb were determined spectrophotometrically using a molar absorption coefficient of 1.71×10^5 M^{-1} cm^{-1} for sperm whale [15] or of 1.88×10^5 M^{-1} cm^{-1} for horse heart [16] at 409 nm.

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Sodium L-ascorbate was purchased from Wako Pure Chemical Industries, Ltd. and used without further purification. All the solutions used for measurements were prepared from redistilled water. A solution of sodium ascorbate was freshly prepared in a nitrogen atmosphere. All other chemicals used were of guaranteed grade.

Kinetic Measurements

All the reactions were carried out in a nitrogen atmosphere as described previously [11]. The reaction was initiated by injecting the solution of sodium ascorbate into the solution containing metMb, NaCl and a Tris-HCl buffer over the pH range from 7.29 to 8.29. The change in absorbance at 550 nm with time was followed with either a Hitachi 200-20 or a Shimadzu UV 140-02 spectrophotometer.

In order to ensure pseudo first-order conditions, sodium ascorbate was in at least a two-hundred fold excess over metMb: the initial concentrations of sodium ascorbate and metMb were 9.79×10^{-3}–0.1 M and $(2.12–3.78) \times 10^{-5}$ M, respectively.

The temperature was controlled at 25.0 ± 0.1 °C and the ionic strength (μ) was adjusted with sodium chloride.

The pH of the solution was measured on a Hitachi-Horiba F-7 pH meter.

Equilibrium Measurements

Acid-dissociation constants of ascorbate ion (HA^-) at different ionic strengths were determined by pH titrations with a standard sodium hydroxide solution at 25 °C in a nitrogen atmosphere. The ionic strength was adjusted with NaCl. The concentration of hydrogen ion was calculated from the pH, using the activity coefficient of H^+ which was evaluated by means of the Davies equation [17]. The solutions of sodium ascorbate (5.00×10^{-3} M) containing NaCl were prepared with air-free redistilled water, and nitrogen gas was bubbled through the cell during each titration.

Acid-dissociation constants of the heme-linked water molecule of metMb were determined spectrophotometrically at 25 °C and at different ionic strengths (adjusted with NaCl). Wavelengths used for measurements were 430 nm, 450 nm, 550 nm, 580 nm, 600 nm and 640 nm. The each solution containing metMb (1.87×10^{-5} M for horse heart and 3.02×10^{-5} M for sperm whale), NaCl and 0.20 M Tris was prepared at a different pH adjusted with HCl (pH 7.27–10.34).

Results and Discussion

Acid-dissociation Constants of Ascorbate

The values of pK_a for the acid-dissociation equilibrium:

$$HA^- \overset{K_a}{\rightleftharpoons} A^{2-} + H^+ \quad (1)$$

are, at 25 °C with ionic strength (M) in parentheses, 11.35 ± 0.03 (0.10), 11.11 ± 0.03 (0.30), 11.03 ± 0.03 (0.60) and 10.91 ± 0.03 (1.0). The value at $\mu = 0.10$ M is in agreement with that of 11.34 determined by Taqui Khan and Martell [18]. The pK_a values decrease with an increase in ionic strength, which shows that the equilibrated fraction of the more negatively charged A^{2-} ion increases with ionic strength.

Effect of Ionic Strength on Acid-dissociation Constants of metMb(H_2O)

The pK values for the acid-dissociation equilibrium:

$$metMb(H_2O) \overset{K}{\rightleftharpoons} metMb(OH) + H^+ \quad (2)$$

are, at 25 °C with ionic strength (M) in parentheses, 8.95 ± 0.04 (0.10), 9.16 ± 0.03 (0.30), 9.26 ± 0.03 (0.60) and 9.35 ± 0.04 (1.0) for sperm whale metMb and 8.82 ± 0.04 (0.10), 8.97 ± 0.03 (0.30), 9.00 ± 0.03 (0.60) and 9.03 ± 0.03 (1.0) for horse heart metMb, respectively. The effect of the ionic strength on the acid-dissociation of the water molecule in horse heart metMb has been studied by George and Hanania [19, 20]. Our results for horse heart metMb are similar to theirs at $\mu > 0.1$ M. These show that an equilibrated fraction of metMb(H_2O) increases with an increase in ionic strength. Sperm whale metMb is more acidic than horse heart metMb.

Determination of the Rate Constants for metMb–ascorbate System

The spectral changes during the ascorbate reduction of horse heart metMb with four isosbestic points at 669 nm, 607 nm, 525 nm and 463 nm were similar to those for the reaction of sperm whale metMb [11]. The reaction product is found to be deoxymyoglobin (the absorption maximum of 556 nm).

Plots of $-\ln(A_\infty - A_t)$ *vs.* time were linear for at least 80% completion, where A_∞ and A_t represent the absorbance at infinity and time t, respectively. The observed first-order rate constants (k_{obs}) obtained from the slope of these straight lines show also a linear dependency on the initial concentrations of ascorbate (Fig. 1). Thus, the rate law:

$$-\frac{1}{2} d[metMb]/dt = k[metMb][HA^-]_0 \quad (3)$$

can be reliable also in the case of horse heart metMb. The second-order rate constants obtained from $k_{obs}/[HA^-]_0$ ($[HA^-]_0$ denotes the initial concentrations of HA^-) increase with a decrease in the acidity (pH 7.29–8.29). The pH dependence of k can be

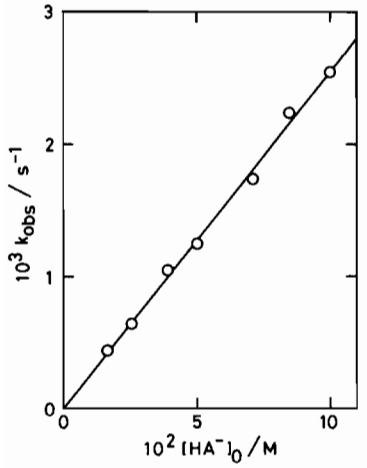

Fig. 1. The dependence of the observed first-order rate constant, k_{obs}, of the reduction of horse heart metMb on the concentration of ascorbate at 25 °C, $\mu = 0.30$ M (NaCl), pH = 7.33 (0.20 M Tris-HCl), 550 nm and $[metMb]_0 = 3.05 \times 10^{-5}$ M.

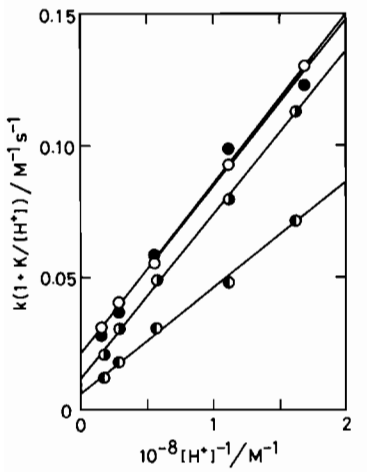

Fig. 2. Plots of $k(1 + K/[H^+])$ *vs.* $[H^+]^{-1}$ for the ascorbate reductions of metMb at 25 °C and 550 nm. $\mu = 0.10$ M (○ horse heart metMb, ● sperm whale metMb) and 1.0 M (◑ horse heart metMb, ◐ sperm whale metMb); $[metMb]_0 = (2.12–3.78) \times 10^{-5}$ M; $[HA^-]_0 = (0.97–1.04) \times 10^{-3}$ M ($\mu = 0.10$ M) and $(4.96–5.12) \times 10^{-2}$ M ($\mu = 1.0$ M); [Tris] = 0.10 M ($\mu = 0.10$ M) and 0.20 M ($\mu = 1.0$ M).

described by the following equations in addition to eqns. (1) and (2).

$$metMb(H_2O) + HA^- \xrightarrow{k_1} Mb + radical \quad (4)$$

$$metMb(H_2O) + A^{2-} \xrightarrow{k_2} Mb + radical \quad (5)$$

$$2radical \xrightarrow{fast} H_nA^{n-2} + dehydroascorbic\ acid \quad (6)$$

TABLE I. Second-order Rate Constants of the Ascorbate Reductions of Sperm Whale and Horse Heart metMb(H_2O) at Different Ionic Strength (NaCl)[a]

Ionic strength (M)	Rate constant			
	Sperm whale		Horse heart	
	$10^2 k_1$ ($M^{-1} s^{-1}$)	$10^{-1} k_2$ ($M^{-1} s^{-1}$)	$10^2 k_1$ ($M^{-1} s^{-1}$)	$10^{-1} k_2$ ($M^{-1} s^{-1}$)
0.10[b]	2.1 ± 0.5	14.0 ± 2.2	2.1 ± 0.1	15.0 ± 0.1
0.30	1.2 ± 0.2	6.9 ± 0.8	1.7 ± 0.3	8.6 ± 1.0
0.60	0.89 ± 0.16	4.1 ± 0.6	1.5 ± 0.2	6.6 ± 0.7
1.0	0.63 ± 0.21	3.3 ± 0.3	1.2 ± 0.2	5.0 ± 0.5

[a]At 25 °C and 0.20 M Tris-HCl buffer. [b]At 0.10 M Tris-HCl buffer.

where the reaction of metMb(OH) with ascorbate is much slower than that of metMb(H_2O) under the present experimental conditions [11]. The above mechanism leads to eqn. (7) for the second-order rate constant:

$$k = \frac{k_1 + k_2 K_a/[H^+]}{1 + K/[H^+]} \quad (7)$$

Plots of $k(1 + K/[H^+])$ *vs.* $[H^+]^{-1}$ gave straight lines (Fig. 2). The second-order rate constants, k_1 and k_2, are obtained from the intercept and the slope of this straight line, respectively, using the K_a and K values. The rate constants, k_1 and k_2, at different ionic strengths are listed in Table I. The value of k_2 for sperm whale metMb at $\mu = 0.3$ M is recalculated from the data given in the previous work [11] with the correction of an ionic strength for K_a of HA^- ion.

Effect of Ionic Strength on the Reduction of metMb-(H_2O) by Ascorbate

The rate constant for the A^{2-} reduction is about 10^4 times larger than that for the HA^- reduction for both systems. The difference in reactivity between A^{2-} and HA^- may be attributed to the difference in their redox potentials; self-exchange rate constants of the couples $HA^{\cdot}/HA^-$ and $A^{\cdot -}/A^{2-}$ are found to be similar ($10^5–10^6$ $M^{-1} s^{-1}$) and the redox potentials of these radicals are estimated as +0.71 V and +0.015 V, respectively [21, 22].

The rate constants for the ascorbate reductions decrease with an increase in an ionic strength, as would be expected for the reaction between opposite charged species: positively charged metMb and negatively charged ascorbate. Figure 3 shows the plots of logarithm of the rate constants, k_1 and k_2, *vs.* the square root of an ionic strength:

$$\log k = \log k_0 + 1.02 Z_1 Z_2 \sqrt{\mu} \quad (8)$$

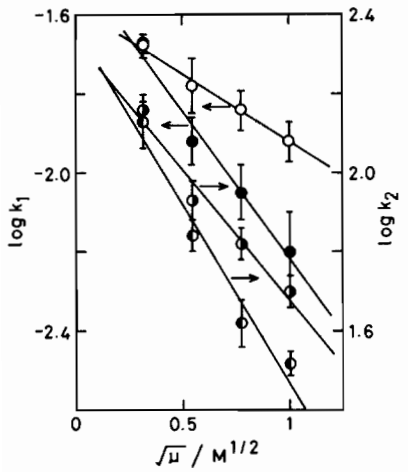

Fig. 3. Plots of logarithm of the second-order rate constants (k_1 and k_2) for the HA^- and A^{2-} reductions of metMb(H_2O) *vs.* the square root of an ionic strength (μ). (○) k_1 for horse heart metMb; (●) k_1 for sperm whale metMb; (◑) k_2 for horse heart metMb; (◐) k_2 for sperm whale metMb.

This relation is applied to the redox reactions of several metalloproteins [23, 24]. Although eqn. (8) should hold only for a dilute solution where μ is small, the linear relation is roughly held in the present case. The effect of an ionic strength on the ascorbate reductions for sperm whale metMb is larger than that for horse heart metMb. The effective charges for sperm whale and horse heart metMb(H_2O) are evaluated as +0.5–+0.7 and +0.3–+0.35, respectively, from the slope of the straight line shown in Fig. 3. These estimated charges for metMbs are reasonable on the basis of the amino-acid sequences, the elution behavior on a CM-cellulose column and p*I* data. From these results the ascorbate reductions of metMbs proceed by the outer-sphere mechanism where there is no specific interaction of ascorbate with metMb.

Figure 3 also shows that horse heart metMb(H_2O) is more reactive than sperm whale metMb(H_2O), although the effective charge of the latter is higher than that of the former. At the higher ionic strength the difference in reactivity is large and at the lower ionic strength it becomes smaller. If the difference in reactivity is only due to the effective charge, sperm whale metMb(H_2O) should be more reactive than horse heart metMb(H_2O). Now we consider the structural difference of both metMbs. In both metMbs the prosthetic group is Fe(III)protoporphyrinIX which coordinates the proximal imidazole of His (F8) and a water molecule, and the heme environment is very similar. Sperm whale metMb has a net of two additional Arg–Asp side chain interactions (Arg45–Asp60 and Arg118–Asp27) [15]. Arg45 also forms a hydrogen bond to the carboxylate of the heme propionate, thereby stabilizing the structure of the heme pocket. In horse heart metMb the Arg residue has mutated to Lys which cannot make simultaneous hydrogen bonds to both the heme propionate and Asp60. It suggests that the heme pocket of horse heart metMb has a more expanded surface compared with sperm whale metMb. Therefore, ascorbate may more easily approach the heme pocket of horse heart metMb than that of sperm whale metMb.

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