The effect of chemical modification of zinc myoglobin on the photoinduced electron transfer with methyl viologen

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Abstract

The chemical modification of zinc protoporphyrin reconstituted myoglobin (ZnPP-Mb) by succinic anhydride gave four kinds of the modified zinc myoglobins (ZnPP-Mb(F2)–ZnPP-Mb(F5)) in which the modification site was different. When ZnPP-Mb(F2)–ZnPP-Mb(F5) were excited in the presence of methyl viologen, photoinduced electron transfer took place between the photoexcited triplet state of zinc myoglobin and methyl viologen to form the separated ion pair. The quenching rate constant by methyl viologen was 3.1, 4.5, 5.4 and 6.6×10^7 M^{-1} s^{-1} for ZnPP-Mb(F2), (F3), (F4) and (F5), respectively. The quenching rate constant was thought to be larger for the sample modified near the specific reaction site.

1. Introduction

Zinc porphyrins are active as photosensitizers and are useful as photochemical probes to study photoinduced electron transfer because they have longer lifetimes at the photoexcited triplet state compared with other metalloporphyrins [1,2]. As the iron ion can be replaced by zinc ion in heme proteins, zinc porphyrin reconstituted heme proteins can be prepared [3–7]. We have previously reported that the photoinduced electron transfer proceeds between zinc protoporphyrin reconstituted myoglobin (ZnPP-Mb) and methyl viologen [7]. When ZnPP-Mb is excited by a laser pulse, the photoexcited triplet state of ZnPP-Mb

(^{3}ZnPP-Mb*) is formed. The quenching of ^{3}ZnPP-Mb* by methyl viologen proceeds with the rate constant of 2.7×10^7 M^{-1} s^{-1} in 10 mM phosphate buffer (pH 7.0) at 25°C to form the separated ion pair [7]. The above results show that zinc porphyrin is also a useful photochemical probe in the biological system to study photoinduced electron transfer.

We have also reported that the quenching rate constant is pH-dependent in the case of the native ZnPP-Mb [7]. The quenching rate constant is drastically changed around at pH 7.8, and increases with increasing pH. This pH-dependence can be explained by the change of lysine and/or histidine residues in their charge with changing pH, i.e., the deprotonation of their residues proceeds by raising pH to increase the negative charge

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of ZnPP-Mb with increasing the electrostatic interaction between methyl viologen and ZnPP-Mb. As the electrostatic interaction accelerates the quenching reaction with methyl viologen, the quenching rate constant increases with increasing pH.

The chemical modification of amino acid residue(s) is a useful method to identify the functional amino acid residue(s) in proteins. Succinic anhydride reacts with lysine and/or histidine residue selectively under alkali conditions. In this work, the chemical modification of ZnPP-Mb by succinic anhydride was carried out, and photoinduced electron transfer was studied for the modified zinc myoglobins to show the effect of the lysine and/or histidine residue at the specific reaction site on the quenching reaction.

2. Materials and methods

Myoglobin from horse skeletal muscle and zinc protoporphyrin IX (ZnPP) were purchased from Sigma Chem. Co., and Porphyrin Products Inc., respectively. DE-52 and DEAE–Sepharose fast flow were obtained from Whatman and Pharmacia Biotech., respectively.

The chemical modification of the myoglobin with succinic anhydride was carried out at room temperature. Succinic anhydride (0.9 g) was added into about 1 g of myoglobin dissolved in 200 ml of 0.5 M Tris–HCl buffer (pH 8.5). Succinic anhydride was added in three portions at intervals of 30 min. The reaction mixture was stirred for 1 h after finishing the addition of succinic anhydride, and then concentrated to about 50 ml by an ultrafiltration membrane (Amicon YM-5). The concentrated solution was dialyzed against 10 mM Tris–HCl buffer (pH 8.5). The dialyzed solution was loaded on a DE-52 column (5×30 cm) previously equilibrated by 10 mM Tris–HCl buffer (pH 8.5). The column was washed by about 900 ml of the equilibration buffer. The adsorbed proteins was eluted by a linear gradient of NaCl, which was 5.00×10^{-4} M min^{-1} (0–100 min) and 2.63×10^{-5} M min^{-1}

(100–2,000 min) at 1.5 ml min^{-1} of the elution rate.

The final separation of F3 and F4 fractions was done by using a DEAE–Sepharose fast flow column (2.5×30 cm). The sample solution was loaded on the column after dialysis and the adsorbed proteins were eluted by a linear gradient of NaCl, which was 5.00×10^{-4} M min^{-1} (0–100 min) and 2.63×10^{-5} M min^{-1} (100–2,000 min) at 0.5 ml min^{-1} of the elution rate.

The chemically modified apo myoglobins [8] and ZnPP reconstituted myoglobins were prepared as reported previously [7]. The concentration of the chemically modified ZnPP-Mb was determined by the absorbance at 280 nm using $\epsilon = 1.58 \times 10^4$ M^{-1} cm^{-1}.

The determination of amino residue was done by using 2,4,6-trinitrobenzenesulfonic acid (TNBS). The apo proteins were prepared from the purified chemically modified ZnPP-Mb according to the method reported in the literature [8]. The prepared apo protein was desalted by using an ultrafiltration membrane (Amicon YM-5). The concentration of the apo protein was determined by the absorbance at 280 nm. 1 ml each of 4% of NaHCO$_3$ and 0.1% TNBS aqueous solutions were added into 1 ml of the sample solution. The reaction was carried out at 40°C for 2 h in the dark, and was stopped by adding 1 ml of 1% SDS solution and 0.5 ml of 1 M HCl. As TNBS reacted with amino residue has the absorption peak at 345 nm ($\epsilon = 1.4 \times 10^4$ M^{-1} cm^{-1}), the absorbance of the reaction mixture was measured at 345 nm to determine amino residues.

The polyacrylamide gel electrophoresis was performed by the method of Laemmli [9], and the gel was stained by Coomassie Brilliant Blue R 250. A Nd–YAG laser was used for laser flash photolysis and the wavelength of the laser pulse was 532 nm. The set-up of the system was same as reported previously [7]. The sample solution was degassed by freeze–pump–thaw cycles before the measurement. The measurement was carried out at 25°C.

3. Results and discussion

When the reaction mixture of myoglobin and succinic anhydride was chromatographed on a DE-52 column, five main fractions (F1–F5) were
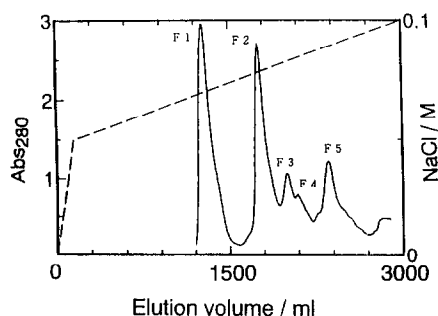

Fig. 1. The elution profile of the chemically modified myoglobins on a DE-52 column. The solid line and the dotted line show the absorbance at 280 nm and the concentration of NaCl, respectively.
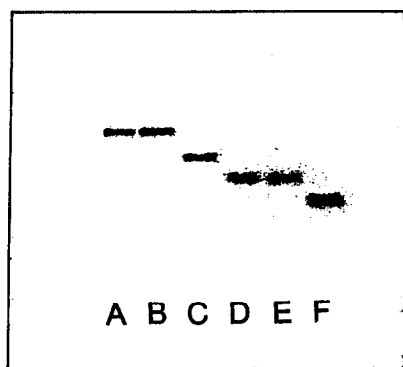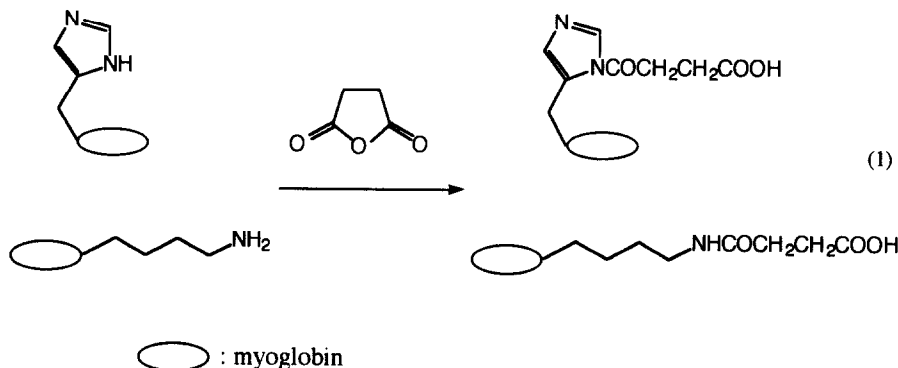

Fig. 2. Polyacrylamide gel electrophoresis of the native and the chemically modified myoglobins. About 15 μg each of the native myoglobin and the sample from F1, F2, F3, F4 and F5 were applied to lane A, B, C, D, E and F, respectively.

obtained as shown in Fig. 1. As the separation of the third and fourth fractions was not enough, these two fractions were combined and rechromatographed on a DEAE–Sepharose column. The purity of the samples obtained from each fractions was checked by polyacrylamide gel electrophoresis (PAGE). All of the samples showed a single band on PAGE as shown in Fig. 2 showing they were purified to electrophoretic homogeneity.

The sample from the first fraction (F1) showed the same mobility on the PAGE gel and the same elution profile on the DE-52 column as the native myoglobin showing that it was non-reacted myoglobin in the reaction. The mobility of the samples from F2 to F5 were higher than that of the native myoglobin. Lysine, histidine and α-amino residue can be modified by succinic anhydride under the experimental conditions. The reactions of succinic anhydride and lysine or histidine residues are shown in Eq. 1 in Scheme 1. When the modification by succinic anhydride takes place, negative charge of the protein molecule increases as shown in Eq. 1 (Scheme 1) to decrease pI value of the protein. The mobility of the sample with lower pI value will be higher on a PAGE gel. Therefore, the result shown in Fig. 2 indicates that the chemical modification took place for the sample from F2 to F5.

The number of the modified lysine residue was determined by using TNBS as described in Materials and Methods for F2–F5. The results are summarized in Table 1, in which the number of the

(1)

◯ : myoglobin

Scheme 1.

Table 1
The number of lysine residues in zinc myoglobins

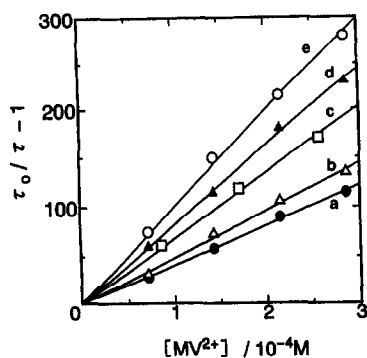| Sample	mol-lysine/mol-Mb
Mb (native)	19.1
Mb(F2)	19.0
Mb(F3)	18.2
Mb(F4)	18.8
Mb(F5)	17.9

Fig. 3. The Stern–Volmer plot for the quenching of the photoexcited triplet state of zinc myoglobin by methyl viologen. 2.0×10^{-5} M each of ZnPP-Mb (a), ZnPP-Mb(F2) (b), -(F3) (c), -(F4) (d), -(F5) (e) and methyl viologen were dissolved in 10 mM phosphate buffer (pH 7.0).

Table 2
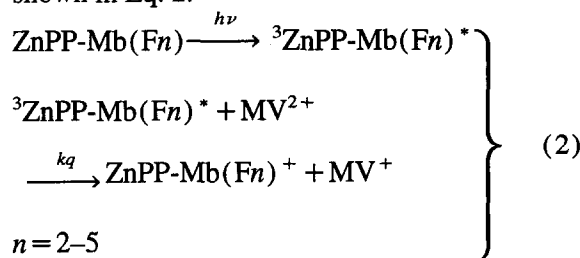The quenching rate constant (kq) and the local charge at the active site (Z_2) of zinc myoglobins at pH 7.0

Sample	$kq/10^7$ M^{-1} s^{-1}	Z_2 [a]
ZnPP-Mb (native)	2.7	−0.47
ZnPP-Mb(F2)	3.1	−0.47
ZnPP-Mb(F3)	4.5	n.d.
ZnPP-Mb(F4)	5.4	n.d.
ZnPP-Mb(F5)	6.8	−3.3

[a] n.d.: not determined

unmodified lysine residues is shown. The native myoglobin from horse skeletal muscle has 19 lysine residues in the molecule. The number of lysines was determined by the TNBS method to be 19.1 for the native myoglobin showing that the TNBS method is reliable for this experiment. The numbers of unmodified lysine residues were 19.0, 18.2, 18.8 and 17.9 for F2, F3, F4 and F5, respectively. This shows that one lysine residue was modified for F3 and F5, and that histidine residue, not lysine, was modified for F2 and F4.

When F2–F5 were used instead of the native myoglobin, the chemically modified zinc protoporphyrin reconstituted myoglobins (ZnPP-Mb(F2)–ZnPP-Mb(F5)) for the samples prepared from F2 to F5, respectively) were obtained. The absorption and the fluorescence spectra of ZnPP-Mb(F2)–ZnPP-Mb(F5) were the same as those of ZnPP-Mb. When ZnPP-Mb(F2)–ZnPP-Mb(F5) were excited at 532 nm by a Nd–YAG laser, the photoexcited triplet state was formed in all cases. ZnPP-Mb(F2)–ZnPP-Mb(F5) showed the identical properties for the photoexcited triplet state, i.e., the decay curves of the T–T absorption obeyed first order kinetics and the lifetime of the triplet state was 15 ms. The lifetime of the photoexcited triplet state of the chemically modified zinc myoglobins was same as that of the native ZnPP-Mb. The above results show that the chemical modification by succinic anhydride did not change the photophysical properties of zinc protoporphyrin in the protein molecules.

When ZnPP-Mb(F2)–ZnPP-Mb(F5) were excited by the laser pulse in the presence of methyl viologen, the quenching of the excited triplet state of zinc myoglobins took place by methyl viologen to form the separated ion pair, showing that the photoinduced electron transfer proceeded also in the chemically modified zinc myoglobins as shown in Eq. 2.

$$\left. \begin{array}{l} \text{ZnPP-Mb}(Fn) \xrightarrow{h\nu} {}^3\text{ZnPP-Mb}(Fn)^* \\[2mm] {}^3\text{ZnPP-Mb}(Fn)^* + MV^{2+} \\[2mm] \xrightarrow{kq} \text{ZnPP-Mb}(Fn)^+ + MV^+ \\[2mm] n = 2\text{–}5 \end{array} \right\} \quad (2)$$

The Stern–Volmer plots are shown in Fig. 3 where all plots show good linear relationship. The quenching rate constant (kq) was determined by the Stern–Volmer plot and the results are summarized in Table 2. The value of kq was 2.7, 3.1, 4.5, 5.4 and 6.8×10^7 M^{-1} s^{-1} for ZnPP-Mb, ZnPP-Mb(F2), (F3), (F4) and (F5), respectively. ZnPP-Mb(F2) showed a similar value of kq to ZnPP-Mb. The value of kq for ZnPP-

Mb(F3)–ZnPP-Mb(F5) was greater than that for ZnPP-Mb in all cases.

The increasing of the quenching rate constant is thought to be caused by increasing negative charge in the modified zinc myoglobin. The increase in negative charge is caused by the chemical modification with succinic anhydride as described above. As methyl viologen is positively charged, the electrostatic interaction between methyl viologen and zinc myoglobin is stronger in the modified zinc myoglobin than the native one to increase the quenching rate constant.

The quenching rate constants for ZnPP-Mb(F2)–ZnPP-Mb(F5) were different from one another. This difference is thought to be caused by the difference of the modification site with succinic anhydride. The local charge around the specific reaction site is important for the photoinduced electron transfer between ZnPP-Mb and methyl viologen [7]. If the chemical modification, therefore, takes place near the specific reaction site, the change in kq will be larger compared with when the modification takes place at a distal site.

To clarify the above hypothesis, the local charge around the specific reaction site was determined. The local charge can be estimated by the Brønsted–Debye–Hückel plot as shown in Eq. 3

$$\log k = \log k_0 + \frac{2AZ_1Z_2 I^{1/2}}{1 + BaI^{1/2}} \tag{3}$$

in which A and B are 0.509 and 0.329×10^8 $M^{1/2}$ cm^{-1}, respectively, in water at 25°C [10]; Z_1 and Z_2 are the charges of methyl viologen and zinc myoglobin, respectively. For Z_1 and a (distance of closest approach between the reactants (center to center)), values of +2 and 2.50 nm were used, respectively. The value of a was assumed by using crystallographic estimation of the sizes of methyl viologen [11] and myoglobin [12] according to the published procedure [13,14].

The plots based on Eq. 3 are shown in Fig. 4 for ZnPP-Mb, ZnPP-Mb(F2) and ZnPP-Mb(F5). All plots showed good linear relationship and the values of Z_2 were determined from the slopes of the lines to be −0.47, −0.47 and −3.3 for ZnPP-
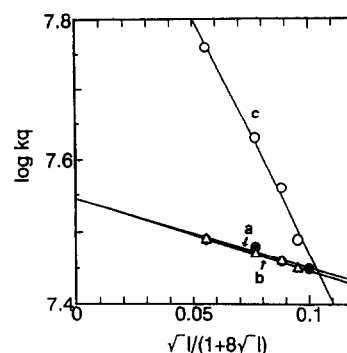

Fig. 4. Plot of $\log kq$ as a function of $I^{1/2}/(1 + 8I^{1/2})$ 2.0×10^{-5} M each of ZnPP-Mb (a), ZnPP-Mb(F2) (b), ZnPP-Mb(F5) (c) and methyl viologen were dissolved in 10 mM phosphate buffer (pH 7.0). The ionic strength was adjusted with NaCl.

Mb, ZnPP-Mb(F2) and ZnPP-Mb(F5), respectively. The local charge at specific reaction site of ZnPP-Mb(F2) was not changed by the chemical modification, but the negative charge increased for ZnPP-Mb(F5). The above results show that the chemical modification took place at a distal site and near the specific reaction site for ZnPP-Mb(F2) and ZnPP-Mb(F5), respectively.

The quenching rate constant was 3.1×10^7 and 6.8×10^7 $M^{-1}s^{-1}$ for ZnPP-Mb(F2) and ZnPP-Mb(F5), respectively. As the chemical modification took place near the specific reaction site to increase the negative charge at the active site in the case of ZnPP-Mb(F5), the electrostatic interaction became stronger to increase the quenching rate constant compared with the native ZnPP-Mb. On the other hand, the local charge at the active site did not change in the case of ZnPP-Mb(F2) because the chemical modification took place at a distal site from the active site. Therefore, the chemical modification did not affect the photoinduced electron transfer in the case of ZnPP-Mb(F2).

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