Chemical Properties of Sperm Whale Myoglobins Reconstituted with Monopropionate Hemins[1]

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The binding behavior of two heme-propionate side chains in sperm whale myoglobin was evaluated using artificially created hemins, 6-methyl-7-propionate- and 6-propionate-7-methyl-protohemin IX. From the thermodynamic study of the hemin binding to apomyoglobin, it was found that two heme-propionates clearly contribute to the stabilization of the hemin in the protein matrix.

Protoheme IX is one of the most popular prosthetic groups in a series of hemoproteins.[2] The heme is bound in the heme pocket via multiple non-covalent interactions such as coordination, hydrophobic contact, hydrogen bonding and so on. In particular, two propionate side chains linked at the 6- and 7-positions of the heme framework exhibit unique hydrogen bonding networks with polar amino acid residues in the protein matrix. In the case of sperm whale myoglobin, two propionates interact with Arg45 and Ser92/His97.[3] Over the past four decades since the elucidation of the 3D structure of myoglobin,[4] the role of each heme-propionate in myoglobin has been discussed using site-directed mutagenesis or replacement of the native heme with a modified one. The latter procedure, modification of the heme-propionates, however, remains limited.[5] We then prepared two monopropionate-mesohemins as artificial hemins in order to obtain an important insight into the relationship between the physiological function and heme-propionate side chains.[1,6] Nevertheless, mesohemin was not an appropriate prosthetic group for the structural and electronic model of the native protohemin. Next, we recently prepared two monopropionate-hemins, **6MeP** and **7MeP**, as an exact model of the prosthetic groups for myoglobin as shown in Chart 1. Although the reconstituted myoglobins with **6MeP** and **7MeP** have been reported by La Mar et al., only the NMR spectra of these proteins were discussed in their paper.[5,7] Here, we report the synthesis of the two hemins, **6MeP** and **7MeP**, and the chemical properties of myoglobins reconstituted with them, particularly, a quantitative analysis of the heme-propionate–globin interaction in the protein matrix.

Monopropionate-hemins, **6MeP** and **7MeP**, were prepared by the slightly modified Smith's method.[8] In the synthetic scheme of **6MeP** and **7MeP**, cyclization of the *a,c*-biladiene salt **1** to give the corresponding porphyrins **2** is one of the key steps. The yield of **2** from **1** was less than 30% under the literature conditions at 145 °C.[8] In contrast, we found that lowering the reaction temperature and dilution of the reaction mixture with DMF prevented the byproduct formation and enhanced the yield to 65–70%.[9] The insertion of the obtained **6MeP** and **7MeP** into apomyoglobin from the sperm whale was carried out using a conventional method.[10,11] The characterization of the reconstituted proteins, rMb(**6MeP**) and rMb(**7MeP**), were carried out not only by NMR but also by ESI-TOF mass spectroscopy. In addition, the UV–vis spectra of rMb(**6MeP**) and rMb(**7MeP**) were completely consistent with that observed for the native myoglobin at pH 7.0.

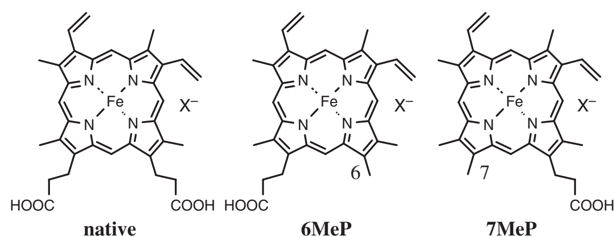
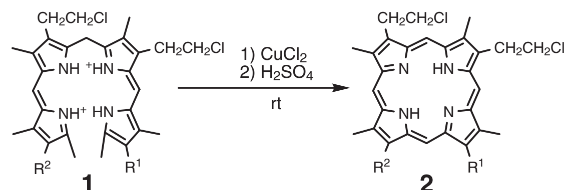


Chart 1.



Scheme 1. R^1 = $-CH_3$ and R^2 = $-CH_2CH_2CO_2CH_3$, or R^1 = $-CH_2CH_2CO_2CH_3$ and R^2 = $-CH_3$.

To evaluate the interaction of **6MeP** and **7MeP** in the protein matrix, the optical spectra of the aquomet form for rMb(**6MeP**) and rMb(**7MeP**) were monitored at various pH values. Two typical spectral changes with clear isosbestic points were observed around pHs 8 and 4. From the titration curve generated from the absorbance changes at 408 nm, we determined the parameters, pK_a and p$K_{1/2}$, which correspond to the pH values upon the deprotonation of the hemin-bound water and release of the 50% hemin from the protein matrix, respectively. Results of the spectrophotometric pH titration for the native and reconstituted myoglobins are summarized in Table 1.

The pK_a values, which demonstrate the acid–alkaline equilibrium constants for rMb(**6MeP**) and rMb(**7MeP**), are clearly shifted in the acid direction by approximately 0.25 pH units compared to that observed for the native protein. It is known that the esterification of the two heme-propionate side chains with meth-

Table 1. pK_a and p$K_{1/2}$ values for native and reconstituted sperm whale myoglobins at 25 °C[a,b]

	Native Myoglobin	rMb(**6MeP**)	rMb(**7MeP**)
pK_a[c]	8.95	8.67	8.73
p$K_{1/2}$[d]	4.16	4.34	4.38

[a]100 mM KCl. [b]Standard deviations in pK_a and p$K_{1/2}$ are all within 0.03. [c]For acid–alkaline equilibrium of aquomet form. [d]pH value upon 50% hemin dissociation from the protein matrix.

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yl groups in myoglobin gives smaller pK_a values than that for native protein, thus, the low pK_as for the distal water ligand in rMb(**6MeP**) and rMb(**7MeP**) results from the same effect observed in the previous studies.[12] Next, the hemin dissociation from the protein matrix was monitored in the lower pH region. The Soret band at 408 nm was replaced with a broad band at 380–385 nm below pH 4 with several isosbestic points, and the latter spectrum is characteristic of the free hemin. From the pH titration curves as shown in Figure 1, the $pK_{1/2}$ values, which correspond to the pH value for the 50% hemin dissociation, were determined. Table 1 demonstrates that the **6MeP** and **7MeP** are released from the protein matrix at ca. pH 4.3–4.4 which is 0.2 units higher than that observed for the native myoglobin. This finding suggests that the lack of the one of the propionates decreases the hemin stability in the heme pocket. In general, the hemin is mainly stabilized by Fe^{3+}–His93 coordination and hydrophobic contact between the hemin and apolar amino acid residues.[13] In addition, Olson and his co-workers suggested that the propionates were not tightly bound to the polar part of the heme pocket, because the hemin dissociation property for myoglobin reconstituted with the protohemin IX-dimethyl ester was very similar to that observed for the native protein.[14] However, in the case of rMb(**6MeP**) and rMb(**7MeP**), we can clearly show that the ionic interaction, i.e., propionate binding to the polar residues, partially stabilizes the hemin in the protein matrix, whereas there is no significant difference in the $pK_{1/2}$ values between rMb(**6MeP**) and rMb(**7MeP**).

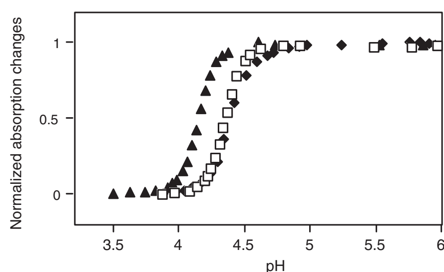

Figure 1. pH Titration of myoglobins: native myoglobin (closed triangles), rMb(**6MeP**) (open squares), and rMb(**7MeP**) (closed diamonds). The Soret maxima at 408 nm were monitored at 25 °C in 100 mM KCl.

To evaluate the apparent hemin affinity for apomyoglobin, we carried out titrimetric measurements by monitoring the spectral changes of the hemins upon the addition of the apoprotein in 100 mM phosphate buffer solutions containing 5% pyridine at 25 °C. Each spectrum was obtained after equilibrium of the hemin insertion into the apoprotein occured (over 30 min). From the spectral changes of the Soret maxima to the 408 nm red-shift with several clear isosbestic points, we determined the apparent free energy changes of the hemin binding for the apoprotein. The ΔG°_{app} values for **6MeP** and **7MeP** are -7.2 ± 0.2 and -7.5 ± 0.2 kcal/mol, respectively, indicating that the replacement of one of the two heme-propionates with a methyl group leads to an unfavorable positive shift of ΔG°_{app} within $\Delta\Delta G^\circ = 1.1$ and 0.8 kcal/mol. Based on these results, we can roughly estimate that two heme-propionate side chains contribute a factor of 10–15% to the overall binding affinity of the native hemin for the protein matrix.[15–17]

Finally, the present study is the first experimental evidence for clearly demonstrating the contribution of two propionates to the stabilization of the hemin in myoglobin. In contrast, no significant difference in the binding property between the 6- and 7-propionates with the corresponding amino acid residues can be detected, although a preliminary study of the myoglobin function suggests that the physiological role of each propionate is clearly different.[18] A detailed study of the ligand binding for rMb(**6MeP**) and rMb(**7Me**) is now in progress.

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References and Notes

1 In this paper, hemin refers to iron(III) porphyrin as a prosthetic group.

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15 It is known that the real affinity of the hemin for apomyoglobin is greater than 10^{10} M^{-1} ($\Delta G^\circ < -13$ kcal/mol). In the present study, the hemin was dissolved in pyridine/buffer solution, because of the hemin solubility. Thus, the apparent binding constants would involve the dissociation factor of the coordinated pyridine from the hemin. However, assuming that the pyridine binding to **6MeP** and **7MeP** are the same as that of the native hemin, we can discuss the difference in the free energy changes between the native and reconstituted myoglobins.

16 La Mar and co-workers have reported that the 6-propionate interaction with Arg45 is stronger by ca. 0.4 kcal/mol than the 7-propionate interaction with Ser92 and His97.[5b]

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