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Sugar-facilitated Incorporation of a Heme Cofactor Bearing Phenylboronic Acid Groups into Apomyoglobin

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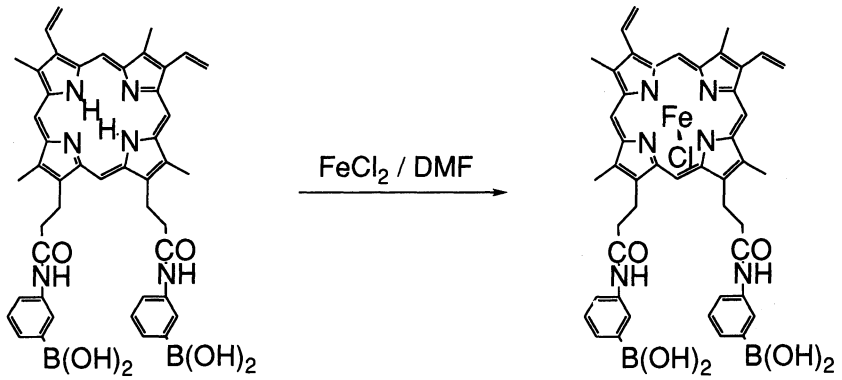
A semi-artificial myoglobin (Mb) with phenylboronic acid pendant groups was synthesized by the cofactor reconstitution method in the presence of sugars. A sugar with higher affinity for phenylboronic acids more efficiently facilitated the Mb reconstitution from a heme bearing two phenylboronic acid groups.

Introduction of nonnatural functional groups to naturally occurring proteins is one of the most promising approaches to give them novel functions. There are a few methods for the synthesis of proteins containing nonnatural molecules at a specific site, such as chemical mutation,[1] total chemical synthesis,[2] semi-synthesis,[3] and in vitro expression of proteins with synthetic suppresser tRNA.[4] We recently proposed that reconstitution of a chemically modified cofactor with apoprotein is valuable for the active-site directed incorporation of various functional groups.[5,6] The reconstitution method successfully provided a photo-activatable myoglobin with ruthenium tris(bipyridine) pendant[5] and a membrane-anchoring protein with a hydrophobic alkyl chain.[6] By using this technique, we tried to incorporate a phenylboronic acid as a sugar recognition site into the proximity of the active site of the myoglobin surface. Here we describe the sugar-facilitated reconstitution of a semi-artificial myoglobin from a heme bearing two phenylboronic acid groups.



2 **1**

Scheme 1.

A synthetic heme appended two phenylboronic acid groups **1** was prepared by iron-insertion of a corresponding free-base porphyrin **2** (Scheme 1).[8] As the first trial, we conducted the reconstitution of **1** with apomyoglobin (apo-Mb, which was prepared from horse heart myoglobin, Sigma Chemical Co.,) according to

the usual manner.[6] The heme **1** (1.2 equiv.) in dimethylsulfoxide (DMSO) was added dropwise to the solution containing apo-Mb (0.2 mM, 1 M = 1 mol dm^{-3}) in an ice bath and incubated at 4 °C for several hours. However, most of **1** was precipitated and as the result, the reconstitution failed. In order to improve the solubility, we tried to add various kinds of sugars to the reaction solution. In this condition, we found that the reconstitution smoothly proceeded without precipitation. This implies that sugars bound to the boronic acid moieties enhance the water-solubility of **1**.

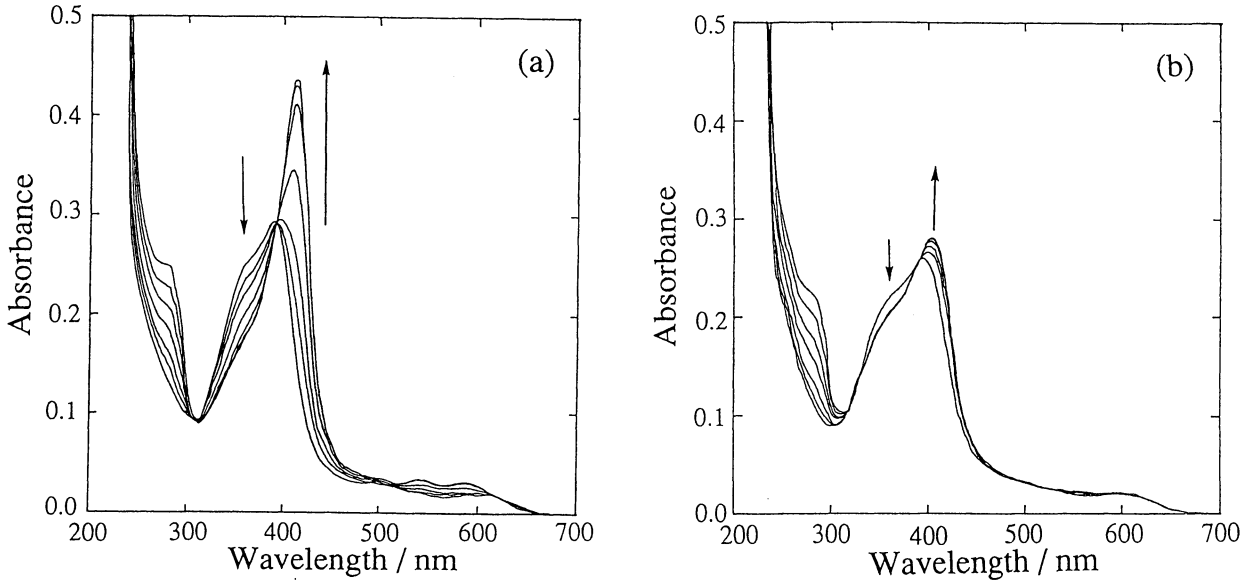


Fig. 1. Absorption spectral changes of the heme **1** by addition of apo-Mb (0 to 1.5 equivalents) in the presence of 0.1 M of D-fructose (a) and 0.1 M of D-glucose (b) (5 min intervals). **1** (**5** μM) in 50 mM carbonate buffer (pH 10.5) containing 0.08%-DMSO.

Figure 1a shows an absorption spectral change of **1** in the presence of D-fructose (0.1 M)[7] by added apo-Mb. The broad Soret-band at 380 nm due to aggregated heme **1** was lessened and the sharp Soret-band at 410 nm gradually intensified with addition of apo-Mb. On the other hands, such a spectral change of **1** did not occur in the presence of D-glucose (0.1 M) and most of the aggregated heme **1** remained as in the case of the absence of sugar (see Fig. 1b).

Figure 2 summarizes spectrophotometric titration curves of **1** with apo-Mb in the presence of various sugars. The spectral change at 410 nm in the presence of D-fructose was saturated at the 1/1 ratio of **1** over apo-Mb, indicating the formation of a 1:1 complex. D-Glucose is not effective to the yield of the reconstituted Mb. The slight change of the UV-visible spectra may be due to the nonspecific adsorption of heme **1** onto the surface of apo-Mb. Only moderate effects are observed when D-mannose and D-arabinose are added to the reaction solution. We recently reported that sugar-binding of a porphyrin **2** induces dissociation of the self-aggregated **2** due to the enhanced hydrophilicity of the porphyrin-sufgar complex.[8] As shown in Fig. 1, the apparent weaker hypochromicity of **1** in the D-fructose solution than that in the D-glucose solution indicates that the looser aggregate of **1** is formed in the presence of D-fructose. Conceivably, the heme deaggregated **1** by the additive sugars could be incorporated into apo-Mb more easily than the tightly self-aggregated **1**. This

idea is supported by the results that the order of the sugar effect in Mb reconstitution (fructose > arabinose > mannose > glucose) is in line with the association constants of phenylboronic acid with various sugars.[9]

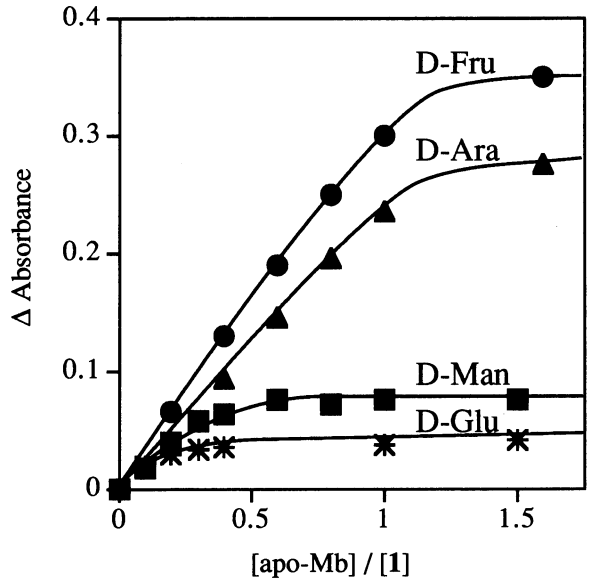


Fig. 2. Spectrophotometric titration curve of **1** with apo-Mb monitored at 410 nm in the presence of sugars. ● D-fructose, ▲ D-Arabinose, ■ D-Mannose, and ✱ D-Glucose.

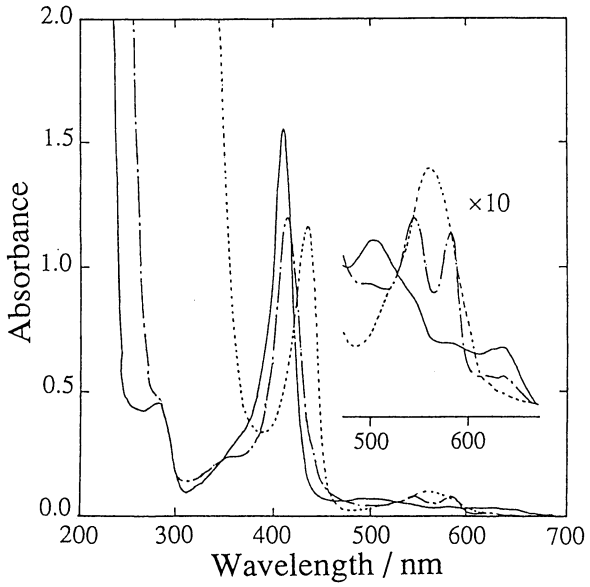


Fig. 3. UV-visible spectra of $Mb(B(OH)_2)_2$ in met-(———), deoxy-(------), and oxy-form(—·—··). $Mb(B(OH)_2)_2$ 10 μM in 10 mM phosphate buffer, pH 6.

The crude reconstituted Mb from **1** ($Mb(B(OH)_2)_2$) in the presence of D-fructose was purified by centrifuge (10000 rpm at 4 °C), dialysis, and gel chromatography (Sephadex G-25). The isolation yield was about 46%.[10] The purified semi-synthetic Mb in oxidized form (met-$Mb(B(OH)_2)_2$) gave a sharp Soret-band at 410 nm and Q-bands at 504 and 630 nm as shown in Fig. 3. Reduction of met-$Mb(B(OH)_2)_2$ by $Na_2S_2O_4$ afforded deoxy-$Mb(B(OH)_2)_2$ (Fe(II)-heme, λ_{max} = 436 and 560 nm), which was then converted to dioxygen complex (oxy-$Mb(B(OH)_2)_2$, λ_{max} = 415, 545 and 582 nm) by bubbling dioxygen gas. The oxy-$Mb(B(OH)_2)_2$ was stable for a few hours at 25 °C under air. Ligand exchange reactions of $Mb(B(OH)_2)_2$ monitored by UV-visible spectroscopy, that is a convenient probe for the microenvironment of the heme in the protein, also show behaviors similar to those of native Mb (azide form of met-$Mb(B(OH)_2)_2$: 423, 545, and 575 nm, fluoride form: 408 and 608 nm).[10] These results demonstrate that the heme **1** is not randomly adsorbed on the apo-Mb surface but incorporated into the active site of myoglobin.

In conclusion, a semi-artificial myoglobin with phenylboronic acid pendant groups is successfully prepared by the cofactor reconstitution method with assistance of sugars. The order of the facilitation effect of additive sugars in the Mb reconstitution is in good correspondence with the order of the affinity of a phenylboronic acid group for sugar. This suggests that the interaction between the heme **1** and apo-Mb is regulated by sugar-binding in the present system. Detailed studies on the structure and the function of the phenylboronic-acid appended Mb are currently under way in our laboratory.

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7) Large excess of sugars are necessary to complexation with phenylboronic acid due to the low binding constants ($10^2 - 10^3$ M^{-1}).[9)]

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10) During the purification processes, the sugar concentration decreased to less than 0.1 mM. Based on the low affinity of phenylboronic acid to sugars,[9)] it is reasonable that the almost sugar is removed after the purification. The yield was determined by absorption spectra of the purified met-$Mb(B(OH)_2)_2$, based on the molar extinction coefficient of the reconstituted Mb from hemin dimethylester ($\varepsilon = 14.4 \times 10^4$ $M^{-1}cm^{-1}$). The yield in the presence of D-glucose was negligible; M. Tamura T. Asakura, and T. Yonetani, *Biochim. Biophys. Acta*, **295**, 467 (1973).

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