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Novel Chemical Modification of Myoglobin by an Alcohol-Responsive Phenylboronic Acid Function

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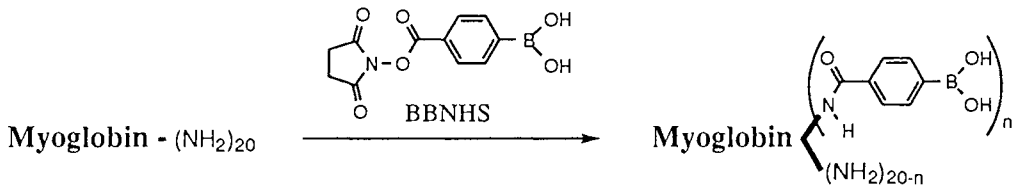
A novel method for modification of myoglobin by an alcohol-responsive phenylboronic acid function has been developed. A modified myoglobin can form a stable complex with poly(vinyl alcohol) in high pH region. The spectroscopic examination indicates that the complexation makes the modified myoglobin more stable against some denaturing factors than native myoglobin.

Chemical modification of proteins that can improve the drawback of proteins, reform their functions, or add new functions has been of considerable attention. [1] Proteins have so far been modified by the covalently-linked water-soluble polymers such as polyvinyl alcohol, [2] polyvinylpyrrolidone, [3] polyethylene glycol, [4] and dextran. [5] However, the more convenient method would be a self-organization system in which proteins are firmly coated through covalent bonds by simply mixing proteins and water-soluble polymers. [6] It is known that boronic acids form cyclic esters with diol groups. The association equilibrium between boronic acids and polyols can be controlled by pH, alcoholic additives, *etc.* Furthermore, this concept will be extended to complexes with polysaccharide, naturally-occurring physiologically-nontoxic "polyols". Here, we report a novel method for modification of a protein (native myoglobin; **native-Mb**) with phenylboronic acids and subsequent self-organization between modified myoglobin and polyols which lead to the improvement of chemical and biochemical stabilities of the protein.

Native-Mb was modified with 4-boronobenzoic acid N-hydroxysuccinimide ester (BBNHS) (Scheme 1). **Native-Mb** has 20 amino groups to be acylated per one molecule. We used one to 5 times equivalent of BBNHS for each amino group. This acylation method using the active ester proceeded more



Scheme1.

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smoothly than the previous one which utilized for the modification of poly(L- and D-lysine)s.[7] The percentage of the modified amino groups in the protein was estimated using the trinitrobenzenesulfonate method.[8] The procedure has established that 15 or 18 amino groups of 20 amino groups are acylated by the use of one or 5 times equivalent of BBNHS (**Mb(15)** or **Mb(18)**, respectively). Modified myoglobin **Mb(15)** was chosen for subsequent investigation because in **Mb(18)** the stability of the oxygen complex was seriously damaged.

Figure 1 shows pH profiles of the absorbance at Soret-band of **Native-Mb** (a) and **Mb(15)** (b) without or with polyols. The absorbance of Soret-band reflects the coordination environment in the heme moiety and further high-dimensional structure in the apoprotein moiety. Among water-soluble polymers used here dextran (average molecular weight; 40000) which contains only trans-diols shows no affinity with boronic acids while poly(vinyl alcohol) (PVA, average molecular weight; 22000) which contains propane-1,3-diol units can interact with boronic acids. In the case of **Native-Mb** no difference was found for the pH profile in the presence or the absence of PVA. On the other hand, when PVA was added, **Mb(15)** showed a small but significant decrease in the Soret-band. These results suggest that at high pH region the binding of PVA takes place and protects the protein from OH^- so that the original structure can be maintained. In order to confirm this hypothesis we measured the circular dichroism (CD) spectra of **Mb(15)** in the presence and the absence of PVA at pH 10, respectively (Fig. 2). In the CD spectrum of **Mb(15)** two negative bands at 210 and 222 nm were intensified in the presence of PVA. This indicates that the α-helix content is enhanced by added PVA.

We also evaluated the influence of a denaturant in addition to pH. Figure 3 shows the stability of proteins against anionic surfactant (sodium dodecylsulfate; SDS) at pH 9.5. The improvement of the stability of **Mb(15)** was found in the presence of PVA. The effect of PVA on the thermal stability was also positive (data not shown).

We hypothesized that the stabilization observed upon addition of PVA to phenylboronic acid-modified myoglobin is due to protection of proteins by polymers against denaturing factors. The change in the molecular

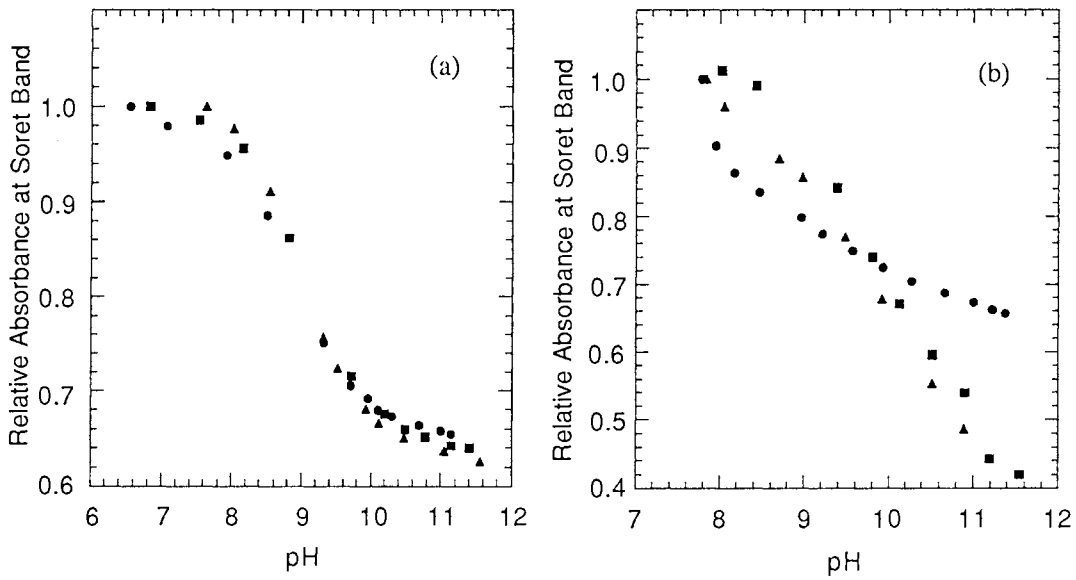

Fig. 1. Effect of pH on the relative absorbance at Soret-band of (a) **Native-Mb** and (b) **Mb(15)**. Protein (1.0×10^{-5} M) at 25 °C in the absence (■) and the presence (0.10 unit M) of PVA (●) or dextran (▲). The pH was adjusted with 1.0 M NaOH .

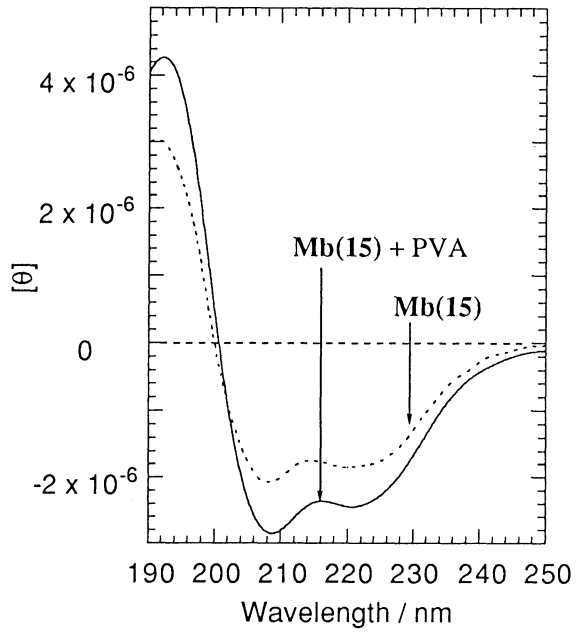

Fig. 2. Circular dichroism spectra of **Mb(15)** (1.0×10^{-5} M) in the presence (0.10 unit M) and the absence of PVA at pH 10 and 25 °C.

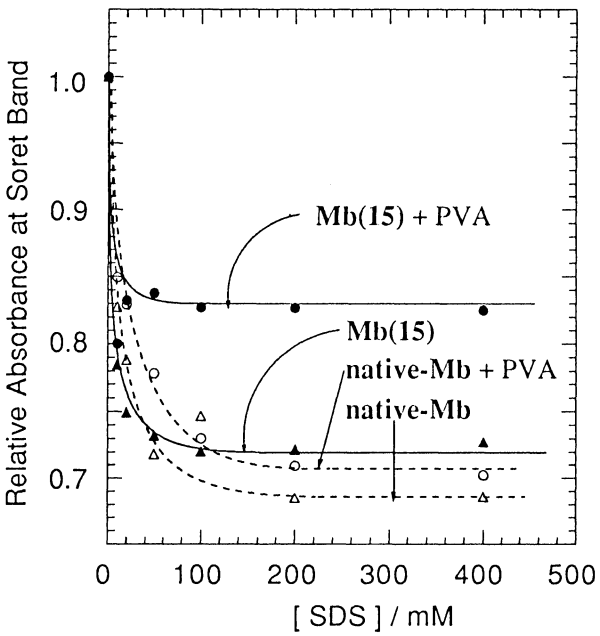

Fig. 3. Stability of **Native-Mb** and **Mb(15)** against SDS in the presence (0.10 unit M) and the absence of PVA at pH 9.5. Protein (1.0×10^{-5} M) in 50 mM carbonate buffer, at 25 °C.

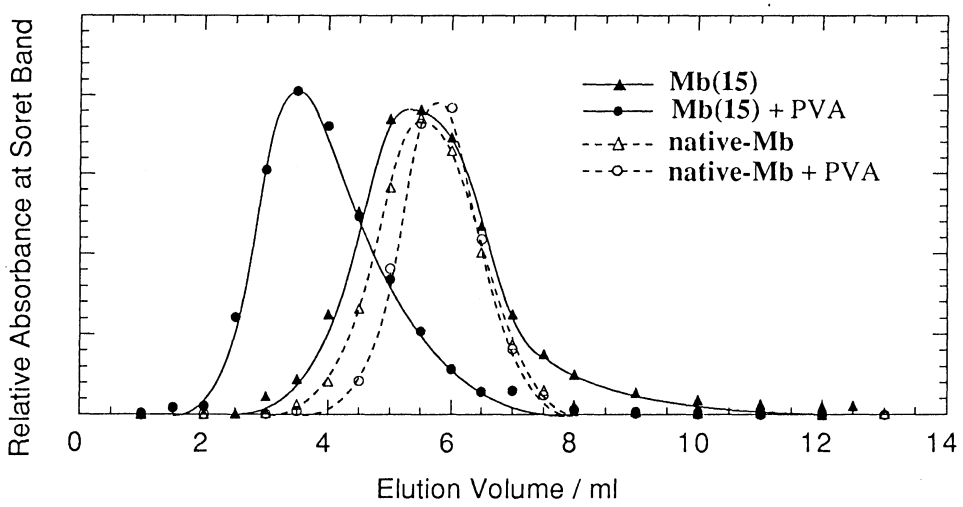

Fig. 4. Hydrodynamic properties of **Native-Mb** and **Mb(15)** in the presence and the absence of PVA at pH 9.5. Samples were individually chromatographed through a Bio-Gel P-100 column.

size (hydrodynamic radii of proteins) which may take place by the interaction between PVA and proteins was studied by size exclusion chromatography (Fig. 4). [9] Unlike **Native-Mb**, **Mb(15)** in the presence of PVA showed an increase in the apparent molecular weight. This suggests that the 1,3-diol unit structure of PVA forms the ester with phenylboronic acids attached to myoglobin and as a result, PVA can efficiently coat the modified myoglobin.

Finally, we evaluated inherent dioxygen binding properties which refect the biolchemical stability of protein in the presence or the absence of PVA at pH 9.5. The dioxygen complexes were prepared by treatment of the proteins with $Na_2S_2O_4$ followed by bubbling dioxygen gas and monitored by a spectroscopic method. The results relating to the half-life periods of auto-oxidation of the dioxygen complexes at 35 °C are summarized in Table 1. Although **Native-Mb** was readily auto-oxidized in a few minutes, **Mb(15)** has a longer half-life time than **Native-Mb** under the similar conditions. Under this pH condition the boronic acid is charged with anion, so that the dioxygen complexes should be stabilized by electrostatic repulsion against replacement by OH^-. Furthermore, since the complexation between phenylboronic acid and PVA is based on the covalent-bonds, the three-dimensional structure would be also stabilized.

Table 1. The half-life periods of auto-oxidation of dioxygen complexes at 35 °C [a)]

	Half-life periods / min			
	None	PVA	D-Fructose	1,3-propanediol
Native-Mb	1.8	1.7	1.0	---
Mb(15)	120	360	132	150

a) [protein] = 1.0 x 10^{-5} M; [D-Fructose] = [1,3-propanediol] = 0.1 M, [PVA] = 0.1 unit M; pH 9.5 buffered with 50 mM carbonate.

In conclusion we succeeded in the improvement of chemical and biochemical stabilities of myoglobin by utilizing a complexation phenomenon between phenylboronic acids and PVA at high pH region. This new method with phenylboronic acids can be applied to other proteins, particularly to those which are expect to work efficiently in high pH region. We are now investigating the possible application of the present system to the stabilization of other proteins.

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9) **Native-Mb** (2.0 x 10^{-5} M) and **Mb(15)** (5.0 x 10^{-5} M) solutions (1.5ml) in 50 mM carbonate buffer solution (pH 9.5) in the presence (0.5 unit M) and absence of PVA were individually chromatographed through a Bio-Gel P-100 (ϕ15 x 50 mm) column with 50 mM carbonate buffer solution as an eluent at 4 °C.

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