Construction of glycosylated myoglobin by reconstitutional method†

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An artificial prosthetic group having β-galactosyl moieties was synthesized and inserted into sperm whale apomyoglobin to successfully afford a glycosylated myoglobin.

Carbohydrate-recognition on a cell surface is one of the key events in various cellular activities, such as fertilization, bacterial/viral infection, cancer transfer, *etc*.[1] The interaction on the cell crucially depends upon the structures of the sugar and glycosylation patterns in the ligand.[2] Therefore, the introduction of an artificially designed carbohydrate interfacing into non-glycosylated proteins, peptides or low-molecular weight compounds will help us to construct a variety of non-natural unique and specific complexes on the cell surface, according to the structures of the installed sugar units. In fact, various proteins and peptides chemically modified with carbohydrate moieties effectively work as inhibitors of the carbohydrate receptors to prevent cellular activities.[3–5] In the case of enzymes, their intrinsic activities are exhibited on the bound cell surface.[5] On the other hand, for the low-molecular weight ligands, metal-containing compounds have been an attractive research target. Especially, porphyrins and their derivatives bearing sugar moieties have been proposed as potential useful probes for medical treatments such as photodynamic therapy (PDT) or cell imaging due to the physicochemical characteristics of the porphyrins.[6–10] Regarding this research trend, we have focused on the interactions of a cell and porphyrin-containing proteins, *i.e*., hemoproteins, in which the hydrophobic prosthetic group is included in the protein matrix.[11]

The introduction of carbohydrate units into a hemoprotein has been attempted by a genetic method although the number of research examples is quite limited.[12] In contrast, another useful strategy would be the reconstitution of a hemoprotein with a synthetic prosthetic group having carbohydrate moieties. The latter approach enables us to readily design structures and the type of conjugation between a heme and the interface.[13] Therefore, in order to study the applicability of this approach, we designed a prosthetic group **1** having galactose moieties, the so-called "galactohemin" (Fig. 1), in which a flexible and branched linker is attached at the heme propionates, and four β-D-galactose units are attached at the other edge of the linker through *N*-glycosyl bonds. The branched linker and introduction of multiple sugar units are essential, because it has been accepted that the multi-valency of carbohydrate ligands remarkably enhances the binding to carbohydrate-recognizing proteins, such as lectins or a cell surface.[14]

The galactohemin **1** was synthesized according to the synthetic route shown in Scheme 1. 2,2′-(*N*-Benzyl)iminodiethanol **2** was reacted with *tert*-butyl acrylate under alkaline conditions, followed by the hydrogenative deprotection of the *N*-benzyl group to afford the secondary amine with ether linkages and *tert*-butyl esters, **3**. After the amine **3** was condensed with *N*-Z-β-alanine (Z = benzyloxycarbonyl) by DCC (DCC = *N*,*N*′-dicyclohexylcarbodiimide), the removal of the Z group gave the linker **4**. The linker was attached to the propionates of protoporphyrin IX in the presence of EDC·HCl (EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) and the *tert*-butyl group was removed under acidic conditions to give **6**. The tetracarboxylic acid **6** was successfully coupled with the *O*-acetylated 1-amino-D-galactose units by BOP ((benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate). In this step, BOP was superior to DCC and EDC·HCl as the condensation reagent. After the iron insertion into the porphyrin–sugar conjugate **7**, the acetyl groups on the galactose units were removed by NaOH/MeOH, and the product was treated with dil. aq. HCl to give **1**. The galactohemin **1** has a high solubility in water and readily forms the μ-oxo dimer in water. The UV-vis bands for **1** in a buffer solution (100 mM phosphate buffer, pH = 7.0) appeared at 360 nm (sh), 396 nm and

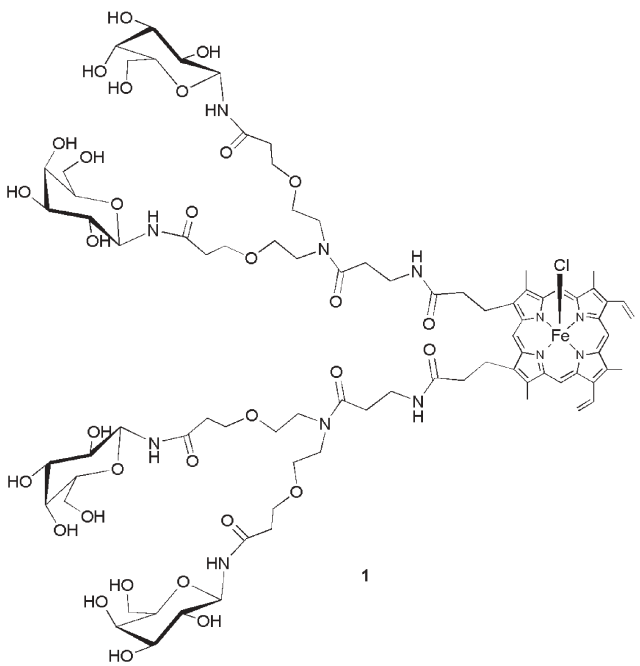

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Fig. 1 Structure of galactohemin **1**.

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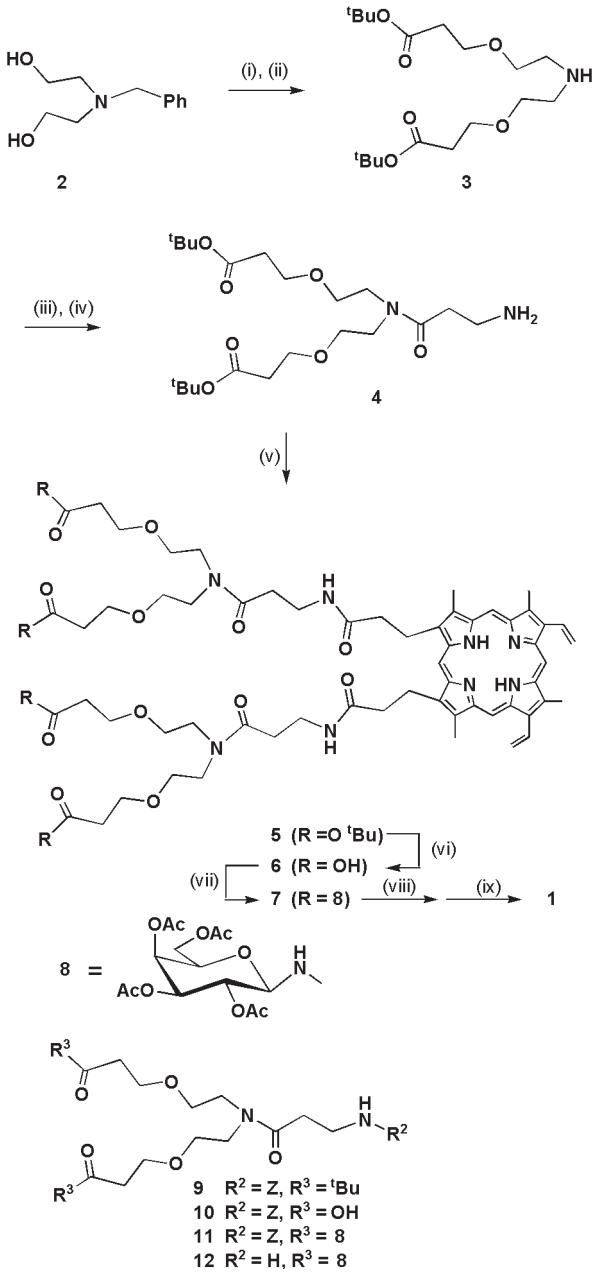

Scheme 1 Synthesis of galactohemin **1**. *Reagents and conditions*: (i) *tert*-butyl acrylate, NaOH, dioxane, r.t., 24 h; (ii) HCO_2NH_4, 10% Pd–C, MeOH, 50 °C, 7 h, 71% in 2 steps; (iii) *N*-Z-β-alanine, DCC, CH_2Cl_2, 0 °C 3 h and r.t. 16 h; (iv) HCO_2NH_4, 10% Pd–C, MeOH, 50 °C, 5 h, 87% in 2 steps; (v) protoporphyrin IX, Et_3N, HOBt·H_2O, EDC·HCl, DMF, 0 °C 2.5 h and r.t. 22 h, 92%; (vi) CF_3CO_2H, HCO_2H, r.t. 22 h; (vii) β-1-amino-2,3,4,6-tetraacetyl-D-galactopyranose, BOP reagent, Et_3N, DMF, 0 °C 3 h and r.t. 40 h, 42% in 2 steps; (viii) $FeCl_2$, K_2CO_3, $CHCl_3$/THF, 50 °C, 24 h; (ix) NaOH, MeOH, r.t. 4 h, 80% in 2 steps.

608 nm, suggesting an equilibrium between the monomer and the dimer under these conditions. As the substituted synthetic routes, one of the possible available routes is that the galactose unit **8** is introduced into the *C*-terminal of the *N*-protected linker **10** to obtain **11** before the attachment of the galactose-pendant linker **12**, the deprotected form of **11** at the *N*-terminal, to the porphyrin propionates. However, we found that the deprotection of the Z-group from **11** by hydrogenation using H_2 on Pd/C was difficult and that the employment of ammonium formate as a hydrogen source was required. After lyophilization of the deprotected amine **12** to remove the excess ammonium salt, it was condensed with protoporphyrin IX by EDC·HCl, but the yield of **7** was unacceptable (less than 20%). This is probably because the high water content of **5** interfered with the smooth coupling of the protoporphyrin propionates.

The ferric galactohemin **1** was smoothly inserted into sperm whale apomyoglobin to afford the glycosylated myoglobin at the terminal of the heme propionates with a yield of *ca*. 40%. When galactohemin **1** in a buffer solution (100 mM phosphate buffer, pH = 7.0) was added to a solution of apomyoglobin, the band at 396 nm shifted to 408 nm, indicating the incorporation of the monomeric galactohemin, followed by the coordination of His93 to the iron atom.[15] The reconstituted myoglobin purified by Sephadex G-25 gel filtration was characterized by electrospray ionization mass (ES-MS) and UV-visible (UV-Vis) spectroscopies. The ES-MS spectrum shown in Fig. 2 demonstrates two multiply charged peaks (8+ and 9+) from the holo form, together with peaks from the apo form and fragmented galactohemin **1** under the current measurement conditions. The deconvolution of the raw spectrum gave the number of 19,065 ± 1, which corresponds to the mass number of the reconstituted myoglobin with **1**. The UV-Vis spectrum (Fig. 3) shows the maximum absorptions at 408, 504 and 632 nm. These characteristic bands are observed in the spectrum of the wild-type myoglobin at almost the same wavelengths, suggesting that **1** is properly accommodated in the heme pocket of myoglobin. The reconstituted myoglobin is stable in a buffer solution (*ca*. 1 mM) at 4 °C for one week. Neither the aggregation nor the release of the galactohemin was observed during this period.

An immunoprecipitation experiment was employed to preliminarily check the function of the galactose units.[16] A mixture of the reconstituted or wild-type myoglobin and commercially available biotin-labelled peanut lectin, a β-galactose-recognizing protein, was treated with streptavidin-modified sepharose. After centrifuging, a

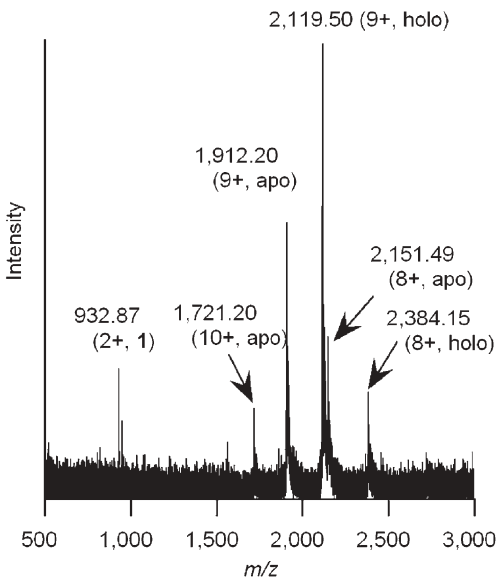

Fig. 2 ES-MS spectrum of the reconstituted myoglobin with **1**.

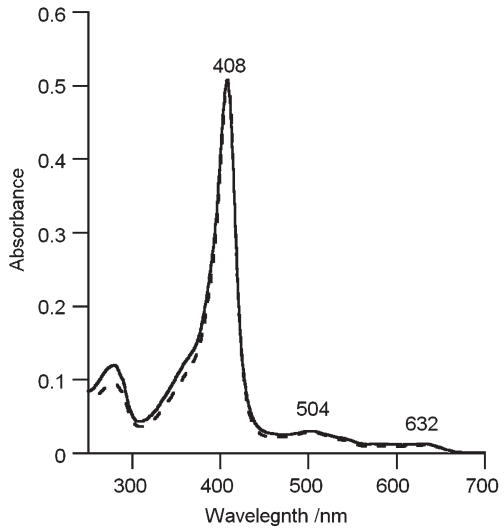

Fig. 3 UV-Vis spectra of the reconstituted myoglobin with **1** (solid line) and wild-type myoglobin (dashed line) in 100 mM phosphate buffer (pH = 7.0).

decrease in the heme content was observed for the solution of the lectin-treated reconstituted myoglobin, whereas such a decrease was not significant for the wild-type myoglobin. This indicates that the galactose units in the reconstituted myoglobin may work as the interface for forming the myoglobin–lectin complex without any non-specific interaction between the myoglobin moiety and the lectin.[17] Further study on the binding of the glycomyoglobin is now in progress, including the optimization of the binding property by investigating the structure of the linker and/or the attached sugar moiety.

In conclusion, the sugar-pendant synthetic heme was successfully incorporated into apomyoglobin to construct "glycomyoglobin". The present study suggests that the reconstitutional method using a synthetic heme is a new strategy to obtain the glycosylated heme protein as well as the previous genetic approach.[12]

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