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Utility of heme analogues to intentionally modify heme–globin interactions in myoglobin☆

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ABSTRACT

Myoglobin reconstitution with various synthetic heme analogues was reviewed to follow the consequences of modified heme–globin interactions. Utility of dimethyl sulfoxide as the solvent for water-insoluble hemes was emphasized.
Proton NMR spectroscopy revealed that loose heme–globin contacts in the heme pocket eventually caused the dynamic heme rotation around the iron–histidine bond. The full rotational rate was estimated to be about 1400 s^{-1} at room temperature for 1,4,5,8-tetramethylhemin. The X-ray analysis of the myoglobin containing iron porphine, the smallest heme without any side chains, showed that the original globin fold was well conserved despite the serious disruption of native heme–globin contacts. Comparison between the two myoglobins with static and rotatory prosthetic groups indicated that the oxygen and carbon monoxide binding profiles were almost unaffected by the heme motion. On the other hand, altered tetrapyrrole array of porphyrin dramatically changed the dissociation constant of oxygen from 0.0005 mm Hg of porphycene-myoglobin to ∞ in oxypyriporphyrin-myoglobin.
Heme–globin interactions in myoglobin were also monitored with circular dichroism spectroscopy. The observation on several reconstituted protein revealed an unrecognized role of the propionate groups in protoheme. Shortening of heme 6,7-propionates to carboxylates resulted in almost complete disappearance of the positive circular dichroism band in the Soret region. The theoretical analysis suggested that the disappeared circular dichroism band reflected the cancellation effects between different conformers of the carboxyl groups directly attached to heme periphery. The above techniques were proposed to be applicable to other hemoproteins to create new biocatalysts.
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1. Introduction

Multiple functions of hemoproteins arise from the exquisite interactions of the heme prosthetic group with globin. The X-ray structural analysis of protein was first reported for myoglobin (Mb) by Kendrew and coworkers in 1958 [1]. Protoheme **1** (Fig. 1), or iron protoporphyrin, is in the hydrophobic pocket to make about one hundred of non-bonded specific contacts with surrounding residues [2]. Mb is one of the most intensively studied proteins, and a large number of tertiary structures of modified and mutant Mbs have been resolved and deposited in the Protein Data Bank. The heme in Mb can be removed, and the resulting apoMb is stable [3,4]. ApoMb couples with various types of artificial cofactors. The protoheme **1** (Fig. 1) in Mb has been consequently replaced with modified hemes to understand the hidden role of **1**.

Tamura and coworkers reported the optical and electron paramagnetic resonance spectra of the Mbs reconstituted with meso-**2**, deutero-**3** or hemato-heme **4** (Fig. 1) [5,6]. They found that the 2,4-heme substituents seriously affect the visible spectral properties. La Mar and coworkers revealed with paramagnetic NMR that heme **1**, which is asymmetric around the α,γ-heme axis (Fig. 1), adopts two orientations in Mb heme pocket [7].

Owing to the advances in porphyrinoid synthesis over these 20 years, a further number of artificial Mbs containing various hemes other than **2**–**4** appeared [8–10]. The new iron porphyrins, when incorporated in the protein pocket, revealed unrecognized aspects of heme–protein interactions. In this article, we review the recent Mb reconstitution works with iron porphyrin analogues, and discuss the possible

Abbreviations: CD, circular dichroism; DMSO, dimethyl sulfoxide; Mb, myoglobin; NMR, nuclear magnetic resonance.

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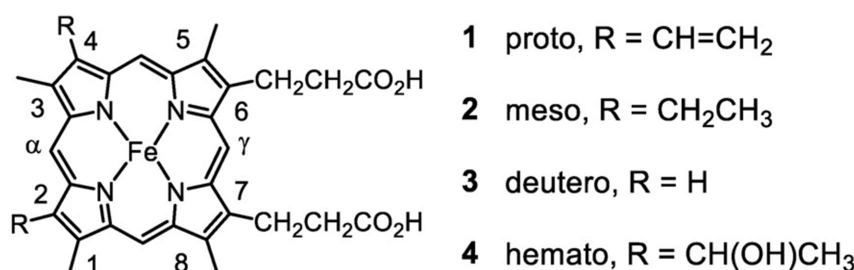

Fig. 1. Structures of protoheme **1** and related hemes **2**–**4** with two propionic acid groups.

application of the results to other hemoenzymes to create novel biocatalysts and signal sensors.

2. Incorporation of water-insoluble hemes into apomyoglobin

The initial step of reconstitution is the separation of protoheme and apoMb. The process is conveniently carried out in large scale with the 2-butanone procedure [3,4]. When the Mb in distilled water is mixed with 2-butanone at pH 2.5, heme **1** (Fig. 1) moves into organic layer, and apoMb is left in water phase. Residual 2-butanone in the aqueous apoMb phase is removed by dialysis against several changes of cold water at pH 7.0. Artificial heme is then added to the apoMb solution. Since natural hemes with free carboxyl groups are soluble in dilute alkaline water, the solvent choice for the heme dissolution is out of problem in ordinary reconstitution process. However, there are many hemes which are totally water-insoluble due to the lack of carboxylic acid groups. How do we dissolve the insoluble hemes into aqueous apoprotein solution? Whenever we deal with insoluble and less soluble hemes, we come up against this problem. We devised a special method to dissolve water-insoluble hemes in water on the protein reconstitution, and reported the technique in the peer-reviewed journal of Japanese Biophysical Society [11]. The procedure reproducibly allowed high-yield preparation of the Mbs containing various all-alkyl hemes. However, the article is written in Japanese, and the experimental details are not readily accessible to non-Japanese researchers. It will be useful to put the main description into English on this occasion.

The protoheme in Mb (100 mg, 5.9 μmol) is removed with the acid 2-butanone method [3], and the crude apoMb in water–butanone mixture is dialyzed overnight to remove the residual butanone against 3 L of 10 mM bis–Tris buffer, pH 6.5, in a cold room. Etiohemin **5** ferric chloride (5.0 mg, 9 μmol; Fig. 2), a typical alkyl hemin without propionate groups, is dissolved into dimethyl sulfoxide (DMSO) $(CH_3)_2S=O$ (2–3 mL) at 120 °C in oil bath. The DMSO solution, cooled to ambient temperature, is titrated over 1 min to stirring apoMb solution of 10 mM bis–Tris buffer (ca. 30 mL) by monitoring pH on ice bath. The mixture is adjusted to pH 6.5 to 6.7 during hemin addition with small amount of dilute hydrochloric acid or aqueous bis–Tris alkaline solution. The volume ratio (DMSO with hemin)/(apoMb solution) less than 0.1 is preferable. The crude hemin–apoMb mixture is dialyzed overnight against 3 L of cold 10 mM bis–Tris buffer, pH 6.5, to remove DMSO before application to a small column (diameter 13 mm, length 60 mm) of Whatman CM-52 cellulose (GE Healthcare, Tokyo) equilibrated with 10 mM bis–Tris at pH 7.0. A linear gradient of bis–Tris buffer, 10 to 100 mM at pH 7.0, is applied in the column, and the main fraction

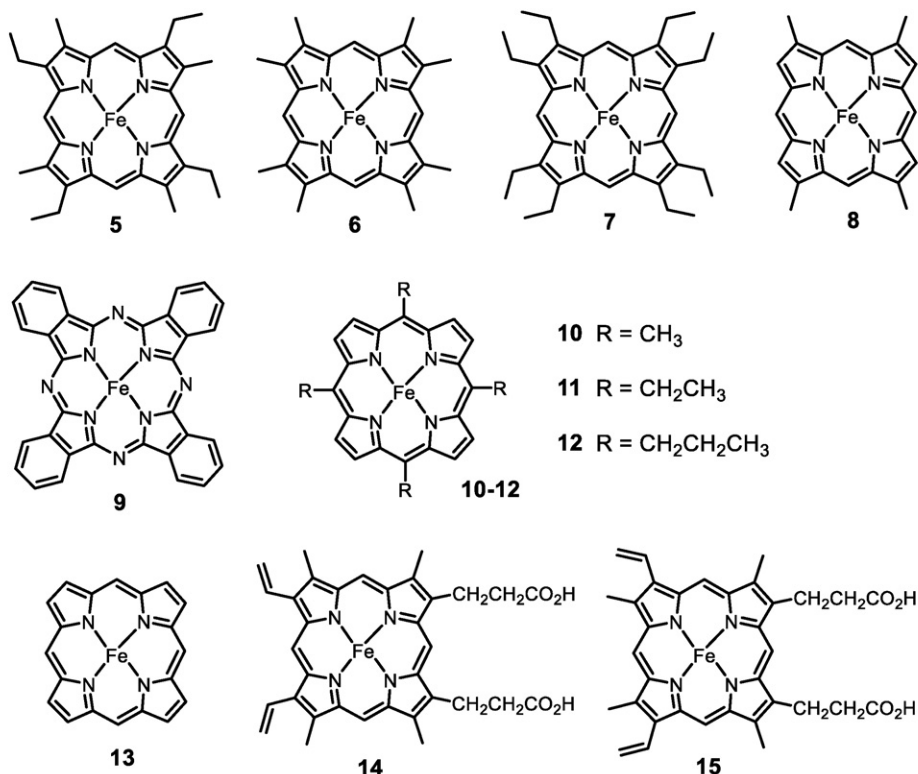

Fig. 2. Structures of heme derivatives **5**–**8**, **10**–**15**, and iron phthalocyanine **9**.

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