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Mössbauer spectroscopic evidence on the heme binding to the proximal histidine in unfolded carbonmonoxy myoglobin by guanidine hydrochloride

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Abstract The unfolded heme structure in myoglobin is controversial because of no chance of direct X-ray structure analyses. The unfolding of carbonmonoxy myoglobin (MbCO) by guanidine hydrochloride (GdnHCl) was studied by the Mössbauer spectroscopy. The spectra show the presence of a sort of spectrum in the unfolded MbCO, independent on the concentration of GdnHCl from 1 to 6 M and the increase of the fraction of unfolded MbCO, depending on the GdnHCl concentration. The isomer shift of the iron of heme in the unfolded MbCO was identified to be different from that of the native MbCO as the globin structure in Mb collapses under the unfolded conditions. This result and the existing related Mössbauer data proved that the heme in the unfolded MbCO may remain coordinated to the proximal histidine.

Keywords Carbonmonoxy myoglobin · Unfolding · Guanidine hydrochloride · Mössbauer spectroscopy · Isomer shift

1 Introduction

Globular proteins have a compact conformation that is essential for their biological function [1]. Proteins are synthesized in cells by a stepwise process in which amino acids are added, one by one, from the NH_2-termini of the chains [2]. The protein

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folding is the process involved in the conversion of an ensemble of newly synthesized (or denatured) polypeptide chain conformations to the unique three-dimensional conformation of the native protein.

Myoglobin (Mb) has its function to store oxygen molecules in the muscles. Its three-dimensional structure as the first protein was determined at atomic resolution [3]. The globin fold is made up of eight helices. The heme in Mb is embedded in a cleft (hydrophobic pocket) made up by the polypeptide chain. The molecular interactions between the heme and the protein are very complex. The heme iron is covalently linked to His93. On the other side of the heme, the distal histidine residue, His64, is one of the important residues in the control of ligand entry. The heme stabilizes intact Mb with respect to its apoMb [4]. The heme binding causes the pocket to collapse tightly around the porphyrin ring, resulting in a more compact protein with an 20% increase in helicity [5].

It has been known that proteins can be unfolded in aqueous solution by high concentrations of certain reagents such as guanidine hydrochloride (GdnHCl) or urea [6]. GdnHCl increases the solubility of the peptide group and almost all of the amino acid chains in aqueous solutions [7]. In the presence of the denaturant, the native state exists in equilibrium with various unfolded states of the molecule. Denaturation with these chemicals is one of the primary ways of measuring the conformational stability of proteins. Conformational transitions of proteins between the folded conformation and the denatured conformation have been observed by a variety of methods, measurements of changes in optical rotatory dispersion, in circular dichroism (CD), in absorption and in fluorescence spectra [2, 6, 8]. Usually, a simple two-state model, $N \rightleftarrows U$, is used to analyze unfolding transitions and the difference in Gibbs free energy between the native (N) and the unfolded (U) states, ΔG. ΔG is assumed to depend linearly on denaturation concentration [9]. The conformational stability of the protein, i.e., the change in free energy between the two conformations in water can be estimated by GdnHCl denaturation curves of proteins. The free energy of unfolding of Mb is reported to be about 10 kcal/mol [10, 11].

The complex interaction between heme and globin in Mb prevents a simple understanding of holoprotein stability. The protein structural deformation upon adding denaturants has been the subject of research. The unfolded heme structure in myoglobin, whether the heme is detached from the protein or the heme is intact even under the denatured condition, is controversial because of no chance of direct x-ray structure analyses. Once if the heme is detached from the protein, the situation is complex. Many studies have been done on the kinetics of heme binding to globin since the pioneering work by Gibson and Antonini [12]. A variety of experimental results shows that the association rate constant of heme and apoMb or apohemoglobin (apoHb) is affected by the states of heme in solution i.e., free heme or nonspecific heme binding to protein (coordination to surface lysines and histidines), monomeric heme or aggregates of heme [12–21]. So far, many experiments on the unfolding of Mb have been done in metMb. Unfolding of MbCO by GdnHCl is known to require extremely long incubation times to reach equilibrium observed by Soret absorbance spectra [14]. Choi and Terajima reported the results of GdnHCl-induced denaturation curves of metMb, deoxyMb and MbCO monitored by the CD method [22]. The association between heme and denatured globin has been postulated as a kinetic intermediate in reconstituting the holoprotein [23]. The possibility that

the heme might remain associated with the denatured protein has been considered in the recent studies [22, 24, 25]. It has been widely believed that the insertion of the heme group is an important posttranslational event in biosynthesis of heme proteins. Much attention has been given to refolding experiments. Chiba et al. [13] chose suitable experimental conditions where the heme exists in monomeric form. Cyanometmyoglobin was unfolded by adding the concentrated urea stock solution where cyanometmyoglobin is fully unfolded and the hemin group is released from the unfolded protein moiety and is expected to exist as a monomeric cyanide-liganded hemin. They found that there are three phases in the refolding process.

In this paper, we will show Mössbauer spectra of the unfolded MbCO by GdnHCl. As far as we know, this is the first time to apply Mössbauer spectroscopy to the unfolding of Mb by GdnHCl. We have chosen MbCO rather than metMb, because of the simple low spin ferrous spectra, a quadrupolar split doublet in MbCO without magnetic splitting [26]. Mössbauer spectroscopy has the merit that it can identify each component of the native MbCO and the unfolded MbCO. Mössbauer spectroscopy measures a number of parameters which can be related to electronic state and bonding type of the iron atom of heme each in the native MbCO and the unfolded MbCO. The effects of the electronic structure on the ^{57}Fe nucleus are transmitted primarily via two hyperfine interactions in Mössbauer spectroscopy: the isomer shift and the electric quadrupole interaction [27]. The isomer shift is highly sensitive to electron density at the nucleus. The calculation of isomer shifts from first principles is difficult, but their usefulness as an empirical parameter. In general covalent ferrous and ferric materials lie in the range of –0.3∼0.3 mm/s (relative to metallic iron). The 14 keV excited state of ^{57}Fe has an electric quadrupole moment, corresponding to an elongation of its charge distribution along the nuclear spin axis. The electric field gradient at the nucleus in sufficiently low symmetry makes the energy shift of the system depending upon the orientation of the nuclear spin.

2 Experimental

Samples ^{57}Fe-enriched protohemin was prepared by the method described by Adler et al. [28]. Native horse heart Mb was purchased from Sigma Chemical Co. Apomyoglobin was prepared by the method of acid acetone and purified by cellulose column. The horse heart Mb solution reconstituted and enriched to 90% with ^{57}Fe was purified according to the method of Ascoli et al. [29] and dissolved in Tris buffer, pH 7.0. MbCO sample was obtained by reducing sperm whale metMb solution with a small amount of $Na_2S_2O_4$ and by introducing CO gas into the mother liquid, and kept under CO atmosphere.

A stock 8 M GdnHCl solution was prepared. In each unfolding experiment the appropriate volume of buffer, stock GdnHCl, and MbCO solution were mixed to achieve the desired GdnHCl concentration (0–6 M) in a final volume of 0.3 cm^3 and to a final concentration of ca. 5 mM MbCO solution. The mixed samples were placed in polyethylene sample holders, and quickly frozen in liquid N_2. The unfolding process was quenched in liquid nitrogen temperature. It took about 15 min before frozen after mixing MbCO and GdnHCl. For the unfolding of MbCO, long time is required to reach equilibrium. Hargrove et al. [14] reported that the stability of Mb is

enhanced 60 fold by reduction of iron to the ferrous deoxy state and 100 fold with CO coordination. They attempted unfolding of MbCO, but extremely long incubation times were required to reach equilibrium, and often oxidation of dissociated CO heme occurred. Our samples in the concentration of GdnHCl between 1 and 5 M may correspond to kinetically be on the way to equilibrium

Mössbauer spectroscopy The Mössbauer experiment was done with a spectrometer of the constant acceleration type and a Kr-gas filled proportional counter. Data for positive and negative acceleration were stored separately in two halves of a multichannel analyzer and then folded. The source used in this experiment was 30 mCi of ^{57}Co diffused in Rh metal. The velocity scale was calibrated by using an iron foil at room temperature and the centroid of the calibration spectrum was taken as zero velocity. In each case the Mössbauer spectra were measured on the samples at liquid nitrogen temperature.

3 Results

The MbCO solution was unfolded by GdnHCl with the concentration of 1∼6 M. Figure 1 shows Mössbauer absorption spectra of MbCO in the presence of 0, 3, and 6 M concentrations of GdnHCl. The spectra in 0 and 6 M of GdnHCl correspond to those of the native MbCO and the unfolded MbCO, respectively. The spectra in 3 M of GdnHCl contain two components of the native and the unfolded MbCO. As the GdnHCl concentration is increased, the fraction of the unfolded component increases. The native MbCO has the quadrupole splitting of 0.36 mm/s and the isomer shift of 0.26 mm/s, independent on the concentration of GdnHCl. The quadrupole splitting of the unfolded MbCO is majorly identical to that of the native MbCO. The isomer shift of the unfolded MbCO is 0.41 mm/s, larger than that of the native MbCO. The quadrupole splitting (0.37 mm/s) and the isomer shift of the unfolded MbCO present small dependence on the GdnHCl concentration. The linewidth of the spectra in the unfolded MbCO increases slightly from 0.22 to 0.27 mm/s between 3.0 and 3.5 M. The Mössbauer spectra were composed of two components of the native MbCO and the unfolded MbCO for the concentrations of GdnHCl, 1 to 5 M. The spectra in the unfolded MbCO showed a sort of spectrum without any other component for the concentrations of GdnHCl, 1 to 5 M. Figure 2 shows the dependence of the mole fraction of the unfolded forms of MbCO on the GdnHCl concentration. The mole fractions were calculated by the ratio of the Mössbauer absorption area of unfolded to the total absorption area.

4 Discussion

Some evidences suggest that the unfolding of metMb results in heme dissociation [14, 23]. However, contrary to these reports, Moczygemmba et al. [25] reported that the unfolding of metMb was reversible (up to 5 M GdnHCl) and no protein concentration dependence was detected for the equilibrium unfolding transition, suggesting that the heme stays coordinated to the unfolding polypeptide. In the

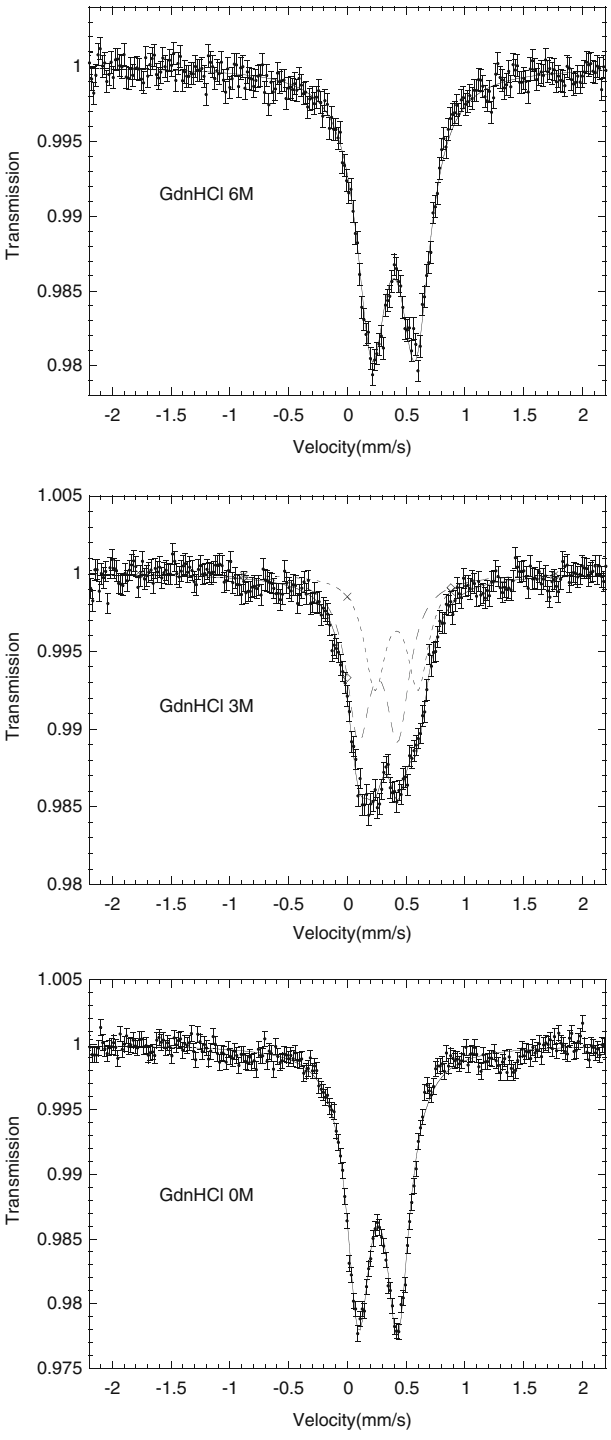

Fig. 1 Mössbauer absorption spectra of MbCO in the presence of various concentrations of GdnHCl at liquid nitrogen temperature. The mixed samples of MbCO and GdnHCl were kept at room temperature during 15 min, and then quickly cooled down to liquid nitrogen temperature. Full curves are calculated spectra based upon parameters values given in the text. *Dashed*, and *dotted curves* show native MbCO and unfolded MbCO, respectively

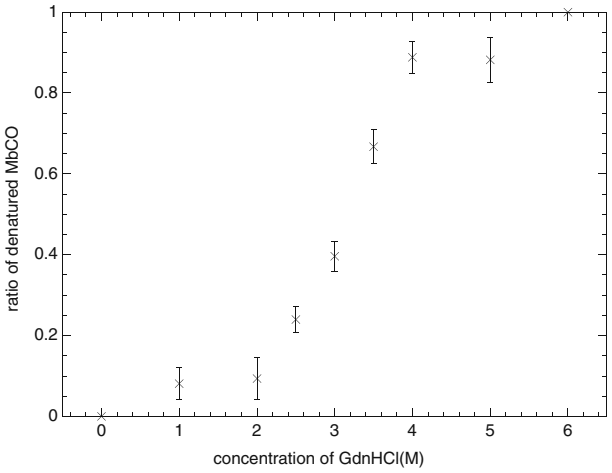

Fig. 2 Dependence of the mole fraction of unfolded forms of MbCO on the GdnHCl concentration

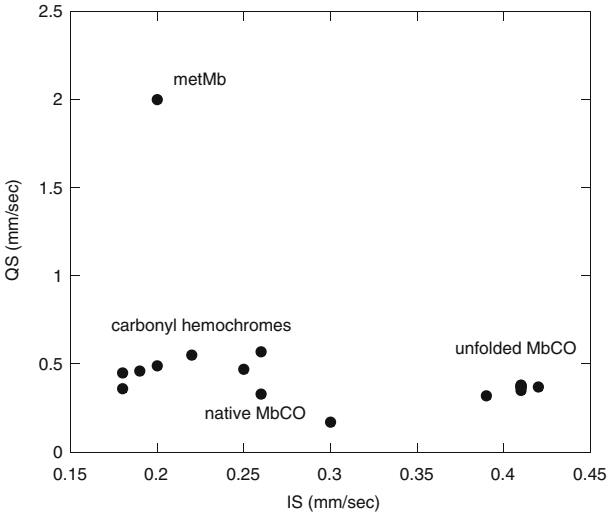

Fig. 3 Mössbauer parameters of the unfolded MbCO, native MbCO, carbonyl hemochromes, and metMb

GdnHCl denaturant experiment by Choi and Terajima [22], the denaturation curve monitored by the circular dichroism (CD) intensity is well expressed by a two-state unfolding transition and any intermediate state has not been observed clearly. The destruction (or deformation) of the secondary structure (α-helices) can be monitored by the CD signal intensity. Changes of the diffusion coefficients of the protein determined by the transient grating (TG) method with the increasing concentration of GdnHCl are almost similar to those measured from the CD signal intensity [22]. They concluded that the heme in MbCO is intact in the protein even under the denatured condition.

If the heme is detached from the protein in the unfolded state, it will be possible either to make an intermediately nonspecific binding of heme-CO to globin [12, 14, 15], and to unfold this intermediate completely to the unfolded state at high GdnHCl

concentrations, or to make heme-CO free. The former case results in a three-state process for the denaturation of MbCO. In our case the spectra of the unfolded MbCO does not basically change up to the high concentration (6 M) of GdnHCl. This is not the case. In the latter case, Connor and Straub [30] have measured the Mössbauer spectra of several carbonyl hemochromes (nonprotein iron porphyrin carbonyls), containing six-coordinated iron (II). The carbonyl hemochromes give sharp, well-resolved quadrupolar split doublets and contain low-spin iron (II). The quadrupole splitting of the unfolded MbCO is in the range of values for carbonyl hemochromes and the native MbCO (Fig. 3), although the isomer shift of the unfolded MbCO is different from that of carbonyl hemochromes. The free heme-CO will form an aggregation of the heme, making dimers involving an oxygen atom bridging two iron ions. The Mössbauer spectra of the porphyrin dimers have been reported that the quadrupole splitting of them are in the range of 0.5∼1.0 mm/s, depending on porphyrin derivatives and temperature [31]. The value is far from that of the unfolded MbCO. From the above discussion, the heme in the unfolded MbCO may remain coordinated to the protein.

The influence of pH on ligand binding to Mb has been studied through His 64 and His93. It is well known that the conformation of the acid-induced denatured Mb is far from a randomly coiled polypeptide but is partially folded, while the strong denaturant such as GdnHCl and urea can induce the complete denaturation of a protein. His93 is involved in the denaturation of Mb at low pH due to its protonation and breakage of the iron-histidine bond. MetMb denatures more rapidly than MbCO at low pH and the rate of denaturation decreases with the increase in CO concentration. The iron-proximal histidine bond breaks in metMb and deoxyMb below pH 4, but remains mainly intact in MbCO down to pH 2.6 [32] by using the resonance Raman spectroscopic method.

Dilg et al. have measured temperature-dependent Mössbauer spectra of a partially unfolded high-potential iron protein [33], indicating an intact metal centre in spite of a protein backbone with a largely collapsed secondary structure. The partial unfolding influences the dynamic properties over the whole physiological temperature regime. The low-temperature Mössbauer dynamics of the folded and unfolded protein are nearly identical with each other. In our paper, at 80 K, the Lamb-Mössbauer factor was assumed to be identical in the folded and unfolded Mb. The ratio of the unfolded Mössbauer absorption area was considered to be the unfolded ratio.

In summary, the spectra show the presence of a sort of spectrum in the unfolded MbCO, independent on the concentration of GdnHCl from 1 to 6 M and the increase of the fraction of unfolded MbCO, depending on the GdnHCl concentration. This experiment proved that the heme in the unfolded MbCO may remain coordinated to the proximal histidine, although the globin structure in Mb collapses under the unfolded conditions.

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