Characterization of Myoglobin Adsorption into Mesoporous Silica Pores by Differential Scanning Calorimetry

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Adsorption of protein molecules into the pores of a porous material is an important process for chromatographic separation of proteins and synthesis of nanoscale biocatalyst systems; however, there are barriers to developing a method for analyzing the process quantitatively. The purpose of this study is to examine the applicability of differential scanning calorimetry (DSC) for quantitative analysis of protein adsorption into silica mesopores. For this purpose myoglobin, a globular protein (diameter: 35.2 Å) was selected, and its adsorption onto mesoporous silica powders with uniform pore diameters (pore diameters: 39 and 64 Å) was measured by adsorption assay and DSC experiments. Our results confirmed that the adsorption of myoglobin into the silica mesopores induced significant changes in the positions and areas of freezing/melting peaks of the pore water. The decrease in heat of fusion of the pore water after myoglobin adsorption could be utilized to quantify the amount of myoglobin inside the silica mesopores. The advantages of DSC include its applicability to small wet mesoporous silica samples.

Keywords Mesoporous silica, myoglobin, adsorption, DSC

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Introduction

Inorganic nanoporous materials have been used in the chromatographic separation of protein molecules and as a solid support for protein encapsulation.1–3 In these applications, the location of protein molecules on the inorganic nanoporous material is a major contributor to the overall separation or biocatalytic reaction.1–3 The key factor is whether or not molecules can adsorb into the pores of the inorganic nanoporous material. The location of the adsorbed protein is, however, difficult to observe, so there is a need for a quantitative characterization method.

Adsorption assays have often been applied to study the adsorption behavior of proteins.2,3 Changes in the concentration of the protein molecules in the supernatant solvent phase are utilized to estimate the total amount of protein adsorbed within the nanoporous material. The total amount of protein adsorbed is used to approximate the adsorption of protein into the pores. Liquid chromatography has also been applied to estimate protein adsorption behavior,1 but it requires the packing of a column with the inorganic nanoporous material. Nitrogen adsorption/desorption isotherm measurement is a more reliable method of observing the location of proteins within an inorganic nanoporous material, since the internal pore adsorption of protein molecules can be confirmed by a decrease in pore volume and average pore diameter.4 The requirement for large sample weights and thermal baking, however, often prevents the application of this technique to adsorption studies of novel protein samples extracted from biological cells. If the size of the inorganic material is greater than a few micrometers, fluorescence microscopy can be applied to observe the distribution of fluorescence-labeled proteins adsorbed within a single nanoporous particle.5 The membrane permeation method is limited to membrane-type inorganic nanoporous materials.6,4 Differential scanning calorimetry (DSC) has been often used to estimate the pore sizes of various nanoporous materials, because the freezing/melting behaviors of water confined inside the nanopore are sensitive to the shape and size of pores.5,11 In the past two decades, the freezing/melting behaviors of pore water have been studied using mesoporous silica materials with uniform and ordered pore structures.11,12 From the results obtained, a modified Gibbs–Thomson model has been proposed to describe the freezing/melting behaviors of the pore water. In the Gibbs–Thomson equation, the freezing/melting temperatures of the pore water are a function of pore size and the interfacial free energies between the pore wall and the inner material of the pore (liquid water and ice).10 The adsorption of protein molecules into the pores decreases the average pore size and includes the contribution of interfacial free energies between the protein surface and the inner material of the pore. In addition, the heats of freezing/melting of the pore water provide information on the amount of pore water, which decreases upon adsorption of protein molecules. It can therefore be expected that the DSC method is useful for examining protein adsorption within an inorganic nanoporous material.

The purpose of this study is to study the applicability of the DSC method for quantitative analysis of the protein adsorption into silica mesopores. For that purpose, SBA-type mesoporous silica (MPS) powders with uniform pore diameters (39 and 64 Å) were prepared. Myoglobin (Mb) was chosen as a model.
protein, since it can retain its globular structure inside the pores of SBA-type mesoporous silica materials. In the present study, the adsorption behaviors of myoglobin within mesoporous silica were examined by adsorption assay, nitrogen adsorption/desorption isotherm, and DSC experiments.

Experimental

Materials and chemicals

Myoglobin (Mb) from equine heart was purchased from Sigma-Aldrich Japan (Tokyo, Japan). The molecular weight of the Mb was 17800 Da. Milli-Q water was used for all experiments. SBA-type mesoporous silica powders were synthesized using Pluronic® P123 or Brij® S10 as a template surfactant. A hexagonal arrangement of one-dimensional pores was confirmed by small-angle X-ray (SAXS) profiles. The SAXS profiles were measured on a Rigaku SmartLab X-ray diffractometer. The pore diameters of MPSs were calculated from the adsorption branch of a nitrogen isotherm using the BJH method (Fig. 1). The structural parameters of MPS powders obtained from the nitrogen adsorption isotherms and SAXS profiles are listed in Table 1. Since SBA-type MPS has micropores within its pore wall, the mesopore volume ($V_{\text{meso}}$) was calculated by subtracting the micropore volume from the total pore volume ($V_p$), which was obtained from the analysis of nitrogen adsorption/desorption isotherms. Hereinafter, we designate MPS as MPS$_{xx}$, where $xx$ means BJH pore diameter in Å.

Myoglobin adsorption assay

The MPS powder (25 mg) was added to 5 mL Mb aqueous solution. The initial concentrations of Mb were between 0.0 mL and 1.5 mg mL$^{-1}$. After being shaken for 20 h, the mixture was centrifuged at 14000 rpm for 10 min. The supernatant was subjected to absorption spectrum measurement to estimate the total amount of Mb adsorbed. The resulting MPS containing Mb was carefully rinsed with water and then suspended in water in preparation for the DSC experiments.

DSC measurement

The MPS powder with or without Mb was dispersed in a small volume of water. The wet MPS sample was put in an aluminum sample pan and sealed with a crimpler. The mass of the wet sample was about 30 mg. DSC measurements were performed on a Rigaku Thermo-Plus DSC-8230 instrument equipped with a cooling system using liquid nitrogen. After the DSC measurement, the sample pan was heated at 200°C for 2 h to measure the weight of the dry MPS sample. The weights of mesoporous silica powder in the aluminum pan were around 4 mg.

Nitrogen adsorption/desorption measurement

The MPS powder (150 mg) was added to 30 mL Mb aqueous solution. The adsorption and rinsing procedures were the same as described above. The MPS powder containing Mb was dried at 90°C in vacuo prior to nitrogen adsorption/desorption measurement using a Micrometrics ASAP 2020 instrument.

Results

Herein, the amount of Mb within MPS as estimated from the adsorption assay is defined as $A_{\text{tot}}$, the total adsorbed amount of Mb. As shown in Fig. 2, adsorption isotherms obtained for MPS39 and MPS64 could be closely fitted to the Langmuir adsorption model. Figure 3 shows DSC curves for MPS powders with and without adsorbed Mb. In the DSC curve for MPS64, the exothermic peak at −22°C and endothermic peak at −15°C are ascribed to freezing and melting of the pore water, respectively (Figs. 3(A) and 3(B)). Both exothermic and endothermic peaks shift to lower temperatures on increasing the amount of Mb adsorbed.

Figure 4 shows the effect of scan rate on the freezing and
melting peaks for MPS64 with Mb. The position of the exothermic freezing peak shifted to a higher temperature on decreasing the scan rate (Fig. 4(A)). In addition, two small exothermic peaks appeared at temperatures ranging from –34 to –40°C when the scan rate was less than 5 K min⁻¹ (Fig. 4(A)). Similar scan rate dependencies were also observed for pore water within MPS64 without Mb. These results suggest that the freezing process of the pore water is influenced by the rate of nucleation and growth of ice crystals inside the silica mesopores. On the other hand, the melting of the pore water was noted as one endothermic peak for every scan rate (Fig. 4(B)). In MPS64 samples with and without Mb, as shown in Fig. 4(C), the position of the onset temperature for melting, \( T_{on} \), was independent of the scan rate. These features of melting peaks suggest that the ice/water melting equilibrium is established inside the pores. Hereinafter, the onset temperature is defined as the melting temperature.

As shown in Fig. 5(A), the adsorption of Mb at MPS64 induces both a lowering of melting temperature (\( T_{on} \)) and a decreasing of the heat of fusion (\( \Delta H \)) of the pore water.
The heat of fusion was estimated from the area of the endothermic melting peak. Since $\Delta H$ is proportional to the volume of pore water, the decrease in $\Delta H$ can be ascribed to the exclusion of pore water accompanied by adsorption of Mb into the pores. The internal pore adsorption of Mb can be supported by the fact of decreasing total pore volume upon adsorption of Mb. As shown in the inset of Fig. 5(A), total pore volume ($V_{pore}$) of MPS64, which was obtained by nitrogen adsorption/desorption isotherm measurement, decreased on increasing the total amount of Mb adsorbed ($A_{tot}$).

The changes in the melting peak found for the MPS39 system are essentially the same as those for the MPS64 system (Figs. 3(C) and 3(D)). The lowering of $T_m$ and decreasing of $\Delta H$ were also noted in connection with adsorption of Mb at MPS39 (Fig. 5(B)).

**Discussion**

The present DSC results confirm that the adsorption of Mb into MPS pores induced freezing/melting temperature depressions of the pore water together with a decrease in the heat of fusion of the pore water. In the freezing/melting temperature depressions, the shift of exothermic freezing peak upon adsorption of Mb is greater than that of the endothermic melting peak (Figs. 3(A) and 3(B)). The position of the freezing peak is thus useful to observe the Mb adsorption into MPS pores. The freezing peak would, however, not be adequate for quantitative analysis of the Mb adsorption, because the change in freezing peak upon adsorption of Mb is complicated (Fig. 3(A)) and depends on the scan rate for the DSC measurement (Fig. 4(A)). The melting peak was recognized as one endothermic peak for each MPS containing Mb (Figs. 3(B) and 3(D)), and did not depend on the scan rate (Fig. 4(C)). These results suggest that an ice/water melting equilibrium is established inside the pores. We therefore focus on the endothermic melting peak for the quantitative analysis of the internal pore adsorption of Mb.

**Change in heat of fusion of pore water after adsorption of Mb**

The MPS has cylindrical mesopore channels and micropores within the silica pore wall (see Fig. 6). Water within the micropores can be regarded as unfreezable due to the small size of the micropores (<2 nm). Inside the mesopore channel, water in the vicinity of the channel wall is also regarded as an unfreezable water layer with thickness $t_{nf}$. These layers do not contribute to the endothermic melting peak on the DSC curve. When the melting peak is due to frozen pore water in the interior of the pore channel, the heat of fusion ($\Delta H$) of the frozen pore water is given by

$$\Delta H = \Delta H_{f,i} m_{MB} V_{meso}(R_{BJH} - t_{nf}/R_{BJH})^2$$

where $\Delta H_{f,i}$, $m_{MB}$, and $V_{meso}$ respectively represent the enthalpy of fusion of water, density of water, weight of MPS, and mesopore volume of MPS. Herein, the BJH pore radius estimated from the nitrogen adsorption branch ($R_{BJH}$) was used.
to define the size of the mesopore channel. The densities of water and ice inside the mesopore channel were assumed to be the same (1 g/cm³). In small angle neutron scattering (SANS) study, the density difference between liquid water and ice was estimated to be less than 8% at below 0°C. The thickness of the unfreezable water layer, \( t_{nf} \), was assumed to be 0.4 nm.

As schematically shown in Fig. 6, the adsorption of Mb molecules excludes both the freezable pore water in the pore interior and the unfreezable water on the pore wall. Herein, the volume fraction of the freezable pore water replaced by Mb molecules is defined by \( \phi \). On the assumption that the \( \Delta H_w \) and \( d_w \) are not affected by Mb adsorption, the change in heat of fusion upon adsorption of Mb, \( \Delta \Delta H(\phi) \), is described by

\[
\Delta \Delta H(\phi) = \Delta H(0) - \Delta H(\phi) = \Delta H(0)(1 - \phi)
\]

With \( \Delta H(\phi) \) values obtained from DSC curves, the \( \phi \) can be estimated by using Eq. (2). When volume of the freezable pore water excluded by one Mb molecule is defined by \( v_{ex} \), volume fraction \( \phi \) of the freezable pore water replaced by Mb molecules is given by

\[
\phi = \frac{A_{pore}N_A}{M_MbV_{meso}} \left( \frac{R_{tot}}{R_{tot} - R_{pore}} \right)^2
\]

where \( A_{pore}, N_A, \) and \( M_Mb \) are, respectively, the amount of Mb inside the pores, Avogadro’s number, and the molecular weight of Mb.

In the MPS39 system, as shown in Fig. 7, \( \phi \) is close to being proportional to \( A_{tot} \); the correlation coefficient is 0.96. This result suggests that almost all the Mb molecules are adsorbed into the pores of MPS39. On the assumption of \( A_{tot} \) being equal to \( A_{pore} \), we calculated \( v_{ex} \) from the slope of the correlation (1.67). The calculated \( v_{ex} \) was 21.9 nm³. Taking into account the geometrical configurations of the freezable pore water, the unfreezable water layer, and spherical Mb (Fig. 6), the calculated \( v_{ex} \) value indicates the radius of Mb spheres to be 1.83 nm. This Mb radius is slightly greater than that of spherical Mb inside pores of MPS39 (1.76 nm), as determined by small-angle neutron scattering (SANS) experiments.13 The hydrated water molecules around Mb are regarded as unfreezable or noncrystalline water. The somewhat large spherical radius suggests that spherical Mb with an unfreezable hydrated water shell excludes the freezable pore water, as shown schematically in Fig. 6.

Our previous SANS study confirmed that the Mb spherical structure is preserved inside MPS pores with pore diameters of 3.9, 7.0, and 7.5 nm. As shown in Fig. 8(A), a marked deviation between \( A_{tot} \) and \( A_{pore} \) is seen for the low \( A_{tot} \) region, whereas it becomes smaller in the high \( A_{tot} \) region. This result indicates preferential adsorption of Mb at the outer MPS64 surface rather than inside the pore. This preferential adsorption behavior is also suggested by the nonlinear relationship between \( A_{tot} \) and total pore volume as determined by the nitrogen adsorption/desorption isotherm (inset in Fig. 5(A)). The Mb molecules initially adsorb at the outer MPS64 surface, so excess adsorption of Mb at the outer MPS64 surface would induce the penetration of Mb into the pores of MPS64.

One plausible reason for the preferential adsorption found with MPS64 is the thickness of the silica pore wall (Table 1). Since the wall thickness of MPS64 (4.5 nm) exceeds the diameter of Mb (3.52 nm), Mb can adsorb to the outer surface of the pore wall. The other reason is the presence of a potential barrier to the penetration of Mb into the pores of MPS64.
Further DSC studies on Mb adsorption to MPS with different pore sizes and wall thicknesses will reveal the preferential adsorption mechanism.

As shown in Fig. 2, the adsorption isotherms obtained by the adsorption assay could be closely fitted to the Langmuir adsorption equation. In the MPS39 system, coincidence between \( A_{\text{ads}} \) and \( A_{\text{pore}} \) is suggested by the DSC experiment, and the adsorption isotherm with \( A_{\text{pore}} \) is also recognized by Langmuir-type adsorption (Fig. 8(B)). This result suggests that almost all of the Mb molecules can enter the MPS39 pores. The preferential adsorption at the outer MPS39 surface would not take place.

In the MPS64 system, on the other hand, the adsorption isotherm with \( A_{\text{pore}} \) appears not to be Langmuir-type adsorption (Fig. 8(A)). This isotherm might be described by the Dubinin-Radushkevich or Dubinin-Astakhov adsorption equation, which is based on the theory of volume filling of small pores by a relatively large adsorbent.\(^{19,20}\)

**Melting temperature depression upon adsorption of Mb**

On the assumption that molar volumes of liquid water and ice are the same, the melting temperature depression, \( \Delta T_m \), of pore water can be represented by the Gibbs-Thomson equation:  \(^{15,16}\)

\[
\Delta T_m = -\frac{2v_w \gamma_w T_w}{R \Delta H_v} \frac{1}{\phi}
\]

where \( v_w \), \( \gamma_w \), \( \Delta H_v \), and \( T_w \) are molar volume of water/ice, interfacial free energy between the pore wall and water/ice, enthalpy of fusion of bulk water, and melting temperature of bulk water, respectively. \( R \) is the effective pore radius, where \( R = R_{\text{BH}} - t_w \). The adsorption of Mb into the pores of MPS decreases the average effective pore radius. The interfacial free energy term is also likely to be affected by Mb adsorption. The Gibbs-Thomson equation thus predicts a shift of melting temperature of the pore water upon adsorption of Mb into the MPS pores.

When the average effective pore size is assumed to govern the melting temperature depression, the change in melting temperature of pore water upon adsorption of Mb is written as follows:  \(^{16}\)

\[
\Delta T_m(\phi) = \Delta T_w(\phi) - \Delta T_w(0) = \Delta T_w(0) \left( \frac{1}{\sqrt{1-\phi}} - 1 \right)
\]

where \( \Delta T_w(\phi) \) and \( \Delta T_w(0) \) are, respectively, the melting temperature depression of pore water in the presence and absence of Mb. \( \phi \) is the volume fraction of Mb inside the pore. Agreement between calculated and observed \( \Delta T_w(\phi) \) values can be seen in the MPS64 system (Fig. 9). On the other hand, the \( \Delta T_w(\phi) \) values observed for the MPS39 system are several times smaller than those calculated using Eq. (5).

Inside the pores of MPS64, there is a small gap between an Mb molecule and the inner pore surface (Fig. 6). The gap size is around 2.7 nm, and water in the gap region can be regarded as unfreezable pore water. The melting temperature of the gap water should be lower than that of water in the interior of the mesopore channel.\(^{10,11}\) The melting of frozen water in the gap region is likely to influence the overall melting equilibrium of the pore water, resulting in melting temperature depression upon adsorption of Mb to MPS64.

In the MPS39 system, since the gap between Mb and the silica pore wall is too narrow (around 0.2 nm), most of the water in this restricted region can be regarded as unfreezable (Fig. 6).\(^{10,11}\) This implies that the water around the narrow gap region contributes only minimally to the total melting equilibrium of the pore water between the Mb molecules. This geometrical configuration of Mb inside the pore channels of MPS39 would thus be responsible for the small melting temperature depression upon adsorption of Mb.

For further elucidation of the relationship between the Mb adsorption and the melting process of the pore water, the pore structures should be precisely determined not only by conventional nitrogen adsorption/desorption isotherm analysis but also by X-ray diffraction or scattering analysis.\(^{21}\) Detailed analysis of small-angle X-ray scattering (SAXS) can provide a reliable picture of pore structure and mesopore volume, both of which are influenced by the synthesis conditions of mesoporous silica. The hydration properties of the Mb inside the pores are also important factor to analyze the Mb adsorption. DSC and X-ray diffraction experiments would be useful for quantitative characterization of the hydration properties.\(^{22}\)

**Conclusions**

In the present study, we demonstrated that the freezing/melting DSC peaks of pore water could be utilized for quantitative analysis of Mb adsorption into silica mesopores. Although the internal pore adsorption of Mb affected both freezing/melting peaks of the pore water, the melting peak was more suitable for elucidating adsorption by the pores. The position (onset temperature for melting) and area (heat of enthalpy) of the melting peak of the pore water could be used in quantitative analyses of Mb adsorption into the silica mesopores.

Since the freezing/melting temperatures of the pore water inside the silica mesopores are far below those of bulk water, the melting/freezing DSC peaks of the pore water and of bulk water can be distinguished. The DSC method can hence be applied to mesoporous silica specimens dispersed in an aqueous solvent. The sample weight used for the DSC experiment was about 4 mg. These are the advantages of DSC over the conventional nitrogen adsorption/desorption isotherm method. The traditional nitrogen adsorption/desorption isotherm technique requires a nitrogen adsorption/desorption isotherm technique requires a large mesoporous silica specimen (about 100 mg) and high-temperature baking prior to the measurement. The DSC method is a potentially useful technique for characterization of the
adsorption behavior of proteins onto inorganic nanoporous materials.

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