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Observation of Fe(III) Configurations of Cytochrome C Confined in Mesoporous Materials

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Abstract

Proteins and enzymes immobilized in confined surroundings of mesoporous materials will develop their structures that are different from those of native solution states. In some cases, the enzymatic activity is shown to be improved when compared to the native solution state. Cytochrome c (cytc) can be used to catalyze the oxidation of some organic substrates in the presence of hydrogen peroxide, i.e. peroxidase-like activity. Immobilization of cytochrome c in mesoporous materials shows improvement of the peroxidase activity. The enhanced activity is reported to be related to the spin configuration of Fe(III) at the active site. In this review, the relationship of the Fe(III) configuration of cytc and the peroxidase activity is discussed. The knowledge on structure-activity of cytc is studied as a model for developing the bio-nano material composites providing better activity of the enzymes.

Keywords: *mesoporous materials, cytochrome c, spin configuration, Electron spin resonance.*

1. Introduction

Immobilization of enzymes and other proteins to inorganic host materials has attracted attention due to many applications in biotechnology and chemical industry such as biocatalysts and drug delivery (1, 2). The discovery of ordered porous mesoporous silica in 1992 by the scientists of Mobil Corporation (3) has offered extended scope of the applications in the host-guess systems at the nanometer scale. The variation of the pore size and geometries can be controlled and provides great advantage over the classical microporous materials, such as zeolytes, to host bulky molecules. To date, the immobilization of small to medium size of enzymes (4, 5) and even a whole cell (6) is possible. The adsorbed biomolecules have been demonstrated to retain their functional characteristics to a large extent. However, the concept of the conjugated materials between bio-molecules and nanoporous materials is in a developing state and there remain many unexplored frontiers from the viewpoints of practical applications and basic science.

To illustrate the detections of structural changes of proteins upon immobilization in the mesoporous silica (MPS), the spin configurations of Fe(III) at the heme group of cytochrome c (cytc) immobilized in different functionalized MPS have been studied. Techniques sensitive to configuration changes of Fe(III) at the active site of the proteins are useful. In this article, a technique called Electron Spin Resonance (ESR) is discussed for investigation of the configuration changes of the ferric ion at the heme active site.

1.1 Structures of mesoporous silica for protein/enzyme immobilization

Mesoporous materials as classified by IUPAC are materials containing pores with diameters between 2.0 and 50 nm. These materials have attracted significant attention due to their high specific surface area, their large specific pore volume and narrow pore size distribution. The pioneer work from researchers of Mobil Corporation was the disclosure of the M41S family of silicate/aluminosilicate mosoporous molecular sieves. They are generally prepared by self-assembly of surfactants and silicates. Here, cationic surfactants possessing alkyl chains from 8 to 22 carbons were used as templates during the hydrothermal synthesis (3) followed by calcination to remove the surfactant template.

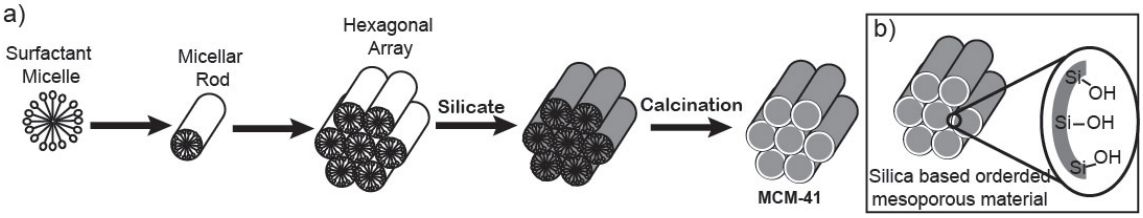

Figure 1. a) General pathway for the formation of ordered mesoporous materials (MCM-41). Adapted from reference (1) by permission from Macmillan Publishers Ltd: Nature.1992;356(6397):710-2, copyright 1992. b) Schematic illustration of ordered mesoporous materials showing high silanol concentration at the surface. Aadapted from Reference (7) with permission of The Royal Society of Chemistry.

Four different phases of have been discovered depending on the shape of the supramolecular template(4); a hexagonal phase such as MCM-41 and SBA-15, a cubic phase MCM-48, a lamellar phase MCM-50, and a cubic octamer [(CTMA) $SiO_{2.5}]_8$ phase. The MCM-41 has been the most primarily studied. The pore size of the materials varies from 2 to 10 nm depending on the synthesis condition, such as the chain length of surfactant, silica source, and temperature range. The general pathway for the formation of the mesoporous materials based on the mechanism proposed by Beck et al, is shown in Figure 1a). The inside of the pore surface of mesoporous materials exhibits high concentration of silanol groups (7) (Figure 1b).

Due to the suitable pore size of mesoporous silica, it has proved to be a good support for various enzymes (8), i.e., the catalytic activity is improved for most enzymes when immobilized in the material. However, there are numerous factors that must be investigated in order to understand the immobilization process and to predict

which enzymes/proteins will adsorb well while retaining their activity and stability. Those factors are, for example; first, characteristics of solid supports, such as surface area, pore size distribution, and surface functional group; second, the compatibility of the size and shape of the host and the enzymes, and isoelectric point (pI) of the enzymes; third, experimental conditions, such as pH, and ionic strength of solutions (4, 8).

It is important that the enzyme is strongly bound to the solid. For pure silica mesoporous solids the attraction between enzyme and the solid surface is relatively weak, and significant leaching of the enzyme away from the surface may occur (5, 8). Modification of the surface with a range of functional groups as linkers can improve the capability for immobilizing the biomolecules. For example, protein adsorption into mesoporous solids is strongly improved in the presence of thiol group on the porous surface (5, 7). The adsorption of cytochrome c, which has high pI at 10.7 (4, 5, 9) and highly positive amino acid residues, can be increased by introduction of aluminum into pure silica materials (10, 11). This is the consequence of the strong electrostatic interaction between the negative charges on the aluminum sites and the positively charged amino acid residues. In addition, the amount of adsorbed protein strongly depends on the size of the solid support. Humphrey *et al* (12) investigated protein adsorption on thiol-functionalized mesopore of 5.1 nm pore size using a series of proteins with molecular weights (MW) ranging from 12,000 to 76,000 Da. The proteins with MW greater than 40,000 Da were excluded from the internal surface while those with smaller size showed significant adsorption. Recently, a series of mesoporous materials with different amount of phenyl group have been synthesized for the immobilization of heme proteins, mainly horseradish peroxidase (HRP) (13). The results showed that the adsorption capacity and peroxidase activity of HRP is higher for the supports with increasing amount of phenyl group on the wall surface. This finding indicates the effect of hydrophobic interactions plays a key role in the enzyme loading and enzymatic activity.

1.2 Structural features of cytochrome c

Cytc is a soluble small heme protein (12,400 Da) and consists of a single polypeptide chain of 104 amino acid residues and iron-containing heme prosthetic group. The active site is located at the heme group which is covalently connected to the peptide chain by two thioether bonds through Cys14 and Cys17. The imidazolyl side chain of His18 serves as an axial ligand to heme iron (Fe), with the second axial ligand from thioether side chain of Met-80. The structure of cytc and coordinates of Fe(III) are shown in Figure 2 obtained from Protein Data Bank code 1AKK (14). It is shown that the double thioether bonds from Cys14 and Cys17 (instead of a single bond) play a role in stabilizing the heme-protein folding and providing sufficiently strong ligand field to favor a low-spin state of Fe(III) (15). The heme group is surrounded with the hydrophobic side chain forming a heme pocket and the heme group is shielded from the solvent. Upon unfolding, heme pocket structural changes occur and result in the replacement of the second ligand by other weak ligands. A high-spin state of Fe(III) is, thus, dominated.

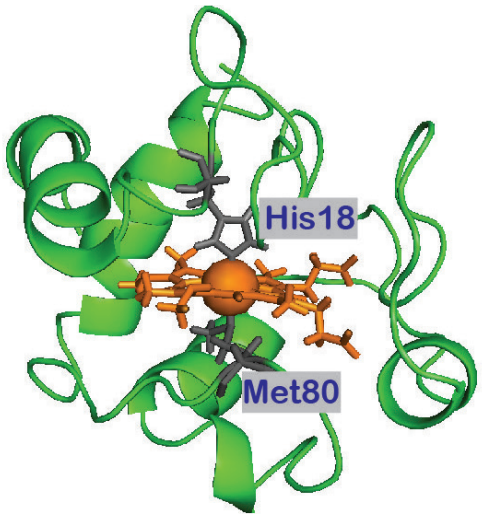

Figure 2. Structure of cyt c showing the protein backbone with α-helices and β-sheet. The heme group is in ball and stick: Fe atom in sphere; Met80 and His18 as axial ligands in ball and stick. The structure is taken from Protein Data Bank code 1AKK (DOI: 10.2210/pdb1AKK/pdb).

2. Enzymatic activity and structural changes of ferric (Fe(III)) cytc

Cytc, an iron-containing heme protein, involves in electron transfer process on the inner mitochondrial membrane. In vitro, the electron transfer behavior of cytc attached on artificial bioeletrochemical devices, i.e., self-assembled monolayer films on gold surface, has been extensively studied (16-18). In addition, cytc is shown as a good biocatalyst (peroxidase-like) for decomposition of hydrogen peroxides (H_2O_2) or organic hydroperoxides, and oxidization of polycyclic aromatic hydrocarbons (PAHs) (19). This activity of cytc is environment-friendly because PAHs have been considered as potential health hazards due to their possible carcinogenic and mutagenic activities. In general, the peroxidase activity can be written as:

$$AH_2 + H_2O_2 \xrightarrow{Heme\ peroxidase} 2H_2O + 2\ {}^{\cdot}AH \quad (1)$$

where AH_2 represents an aromatic hydrocarbon as a one-electron donor compound and ${}^{\cdot}AH$ is the corresponding radical (20).

The enzymatic activity of cytc immobilized in confined environments, such as porous membrane of MPS materials (10, 11, 21, 22) and anionic charged surfactants (23-25), is shown to be improved with respect to cytc in solution. Furthermore, the porous structure of the MPS solids can protect the protein from thermal degradation. It is believed that structural changes of cytc caused by the enclosed surroundings, especially those which are negatively charged, facilitate the reaction of H_2O_2.

The heme ferric iron plays an important role as a catalytic center in cytc. Lee *et al*(11) investigated the relationship between peroxidase activity and spin configuration of Fe(III) of cytc in different environments of MPS. Figure 3 shows Electron Spin Resonance (ESR) spectra of cytc incorporated in two modified MCM samples, i.e. aluminosilicated MCM-48 (Al-MCM48) and thiol-functionalized MCM-41 (SH-MCM41). The negative charge on the surface of Al-MCM48 induces electrostatic interaction to cytc whereas the thiol group on that of SH-MCM41 develops covalent bonding to the Fe(III) active site of ctyc. The ESR spectra show the signals of Fe(III) at different configurations as followed: high spin at g = 6.0 and 1.9-2.0, and low spin at g = 2.3. It should be noted that the signal at g = 2.0 not only contributes to high spin Fe(III) but also to the protein radical intermediate generated during the reaction. In addition, free Fe(III) is detected at g = 4.3 which is the result of the breakdown of the heme Fe(III) at active site. Figure 3a shows the signal for the Al-MCM48/cytc sample at g-values of 6.0 and 1.9 which are assigned to high-spin Fe(III) configuration. After treated with H_2O_2, the signal at g = 6.0 is decreased, most likely due to the oxidation of Fe(III) to Fe(IV) heme. Furthermore, the appearance of g = 4.3 and 2.0 indicating the free Fe(III) species and protein radical, respectively, as shown in Figure 3b. For the SH-MCM41/cytc, a mixture of high and low-spin species is found (Figure 3c) as appeared in the following signals: g = 6.0 and 2.0 (high spin), g = 2.3 (low spin), and g = 4.3 (free Fe(III)). After H_2O_2 treatment, the intensity of high-spin and low-spin states decreases, but that of high-spin decreases more (Figure 3d). In general, the spectral feature after treated with H_2O_2 of both Al-MCM48/cytc (Figure 3c) and SH-MCM41/cytc (Figure 3d) is analogous. The high-spin Fe(III) observed in both immobilized cytc samples suggests that the axial Met-80 ligand may be replaced by H_2O or a silanol group on the silica surface. The low-spin Fe(III) observed in SH-MCM41/cytc indicates the thiol group coordination to Fe(III).

To relate the peroxidase activity to spin configuration of heme Fe(III), the peroxidase activity was assayed based on Equation 1 using pyrene as an aromatic hydrocarbon. The SH-MPS-cytc sample exhibits lower activity than the Al-MPS-cytc sample (specific activity of 0.01 vs. 0.18 min^{-1}). The result from ESR showed that the portion of low-spin state is higher in SH-MPS-cytc than that in Al-MPS-cytc. The authors, therefore, proposed that the decrease in catalytic activity of the low-spin configuration is a result of the coordination of the thiol group with the Fe(III). The strong bonding of –SH to Fe(III) poisons the active site of cytc. On the other hand, the enhanced activity in a high-spin configuration is attributed to a fully open heme groove and, thus, more accessible to active site. However, the activity of SH-MCM-cytc was found higher than that of native cytc in solution, which contains

only low-spin Fe(III) heme.

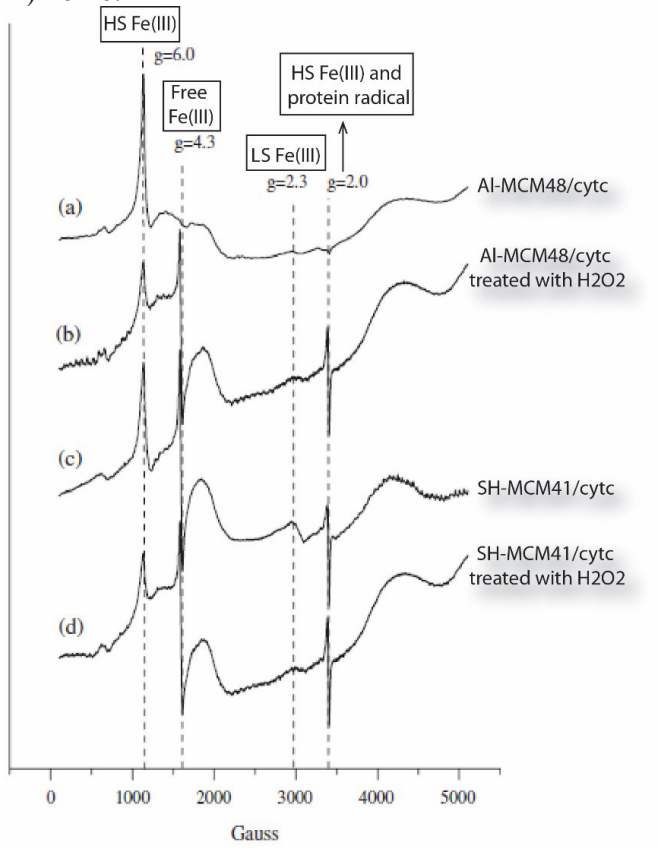

Figure 3. ESR spectra at 10 K of the following samples: (a) Al-MCM48/cytc, (b) Al-MCM48/cytc treated with H_2O_2, (c) MCM41-SH/cytc, and (d) MCM41-SH/cytc treated with H_2O_2. The Version of Scholarly Record of this article is published in Reference (9): Mol. Phys. 2006;104(10-11):1635-41.
Abbreviations: HS = high-spin, LS = low-spin.

Figure 4 illustrates the conformational changes in the peptide chain of ferric cytc immobilized in Al-MPS and SH-MPS and compare to the known native structure in solution. The catalytic activity of cytc in different environment is increasing as following: in solution (low spin Fe, S=1/2) < Al-MPS (mixture of high spin, S=5/2, and low spin, S=1/2) < SH-MPS (high spin, S=5/2), where S is the spin quantum number of Fe(III). In summary, The high-spin Fe(III) involves H_2O or a silanol group at the axial ligand implying that the Fe(III) active site is more exposed to solution allowing more accessible to the substrates.

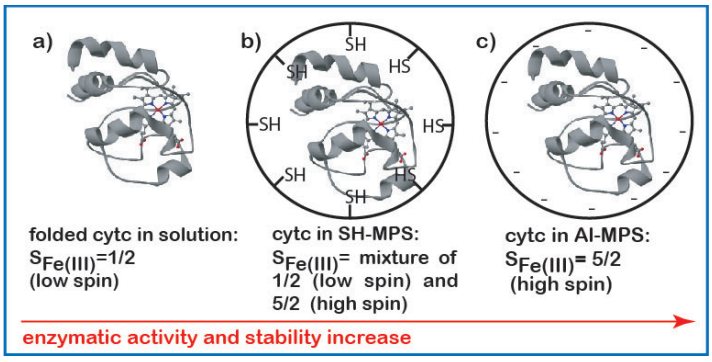

Figure 4. Schematic representation of electron configuration of heme Fe(III) in different environments. a) in solution, b) in SH-MPS, and c) in Al-MPS. The scheme is depicted based on the results from Reference (9): Mol. Phys.2006;104(10-11):1635-41.

Recently the catalytic activity of cytc is studied in a variation of functionalized MPS including mesoporous silica sheet which shows higher activity relative to cytc in MCM-41 and SBA-15 (26). In addition, the relation between the peroxidase activity and the structural change of cytc when confined in MPS was observed (27). The orientation of cytc was examined in the surface-modified cylindrical MPS with three different linkers to ensure different environments at the Fe(III) active site by covalent bonding. The relation of spin configuration and activity is consistent with the previous work, i.e, the high-spin state showed higher peroxidase activity relative to the low-spin state.

3. Conclusions

Proteins submitted to restricted environments in mesoporous materials develop conformational changes with respect to their native folded states. The peroxidase activity of folded cytc in solution is very poor; however, it is dramatically increased upon unfolding. It is known that the structural changes at the heme group and in its vicinity play an important role in activating the peroxidase activity. Intermediate non-native folded states of cytc have been determined upon interactions to anionic inner pores of mesoporous materials implying conformational heterogeneity of the protein structures. The structural heterogeneity and dynamics of the protein complexes, however, have caused challenges to many spectroscopic techniques.

The data from ESR showed that the high-spin state of Fe(III) relates to higher activity of cytc when confined in MPS such as Al-MCM41 which is the result from the electrostatic interaction between cytc peptide residues and the inner surface of the MPS. The interaction induces more opening of hydrophobic region at the ferric iron heme. On the other hand, the functionalized MPS that binds covalently to the Fe(III) active site develops partial low-spin state ferric heme which shows lower peroxidase activity. The modification of the functional group on silica surface is, therefore, important in controlling the environment around the Fe(III) heme. The knowledge of the

structural change of the protein at the active site is significantly important for the design of MPS material serving for maximal activity of the enzyme.

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