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Catalytic oxidation of *o*-phenylenediamine by cytochrome *c* encapsulated in reversed micelles

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Abstract

Cytochrome *c* solubilized in reversed micelles formulated with di-2-ethylhexyl sulfosuccinate (AOT) has allowed to catalyze the oxidation of *o*-phenylenediamine (*o*-PDA) with hydrogen peroxide. The oxidative reaction did not occur effectively by cytochrome *c* in water. The nanostructural environment in reversed micelles seemed to perturb the three-dimensional structure of the active site of cytochrome *c*. The product obtained in reversed micelles had a specific absorption spectrum with the absorption peak at 720 nm, indicating a blue-colored solution, whereas common peroxidases such as horseradish peroxidase provided a yellow-colored product in water with the absorption peak at 420 nm. The difference in the oxidative products suggests that AOT molecules play an important role in the specificity of *o*-PDA oxidation. The dependency of the product species on the pH of the inner droplet in reversed micelles indicated that the specific oxidative product was prepared by electrostatic interaction with AOT molecules.

Keywords: Cytochrome *c*; Reversed micelles; Peroxidase; Phenylenediamine; Oxidation

1. Introduction

Peroxidases catalyze one-electron oxidation of various organic substrates. Such function has a great potential for biotransformation, indicating a precisely designed production through an enzymatic conversion. Recent studies concerning enzymatic oxidation have attracted considerable attention [1]. In particular, peroxidases in organic media were found to catalyze enzymatic polymerization of hydrophobic substrates (monomers) [2]. The phenol oxidation leads to a phenolic resin, which is an industrial important polymer, and the aniline oxidation provides polyaniline as an electron-conducting polymer [3].

It is well known that reversed micelles facilitate solubilization of biomolecules such as enzymes (proteins) [2] and nucleic acids [4] in organic media. Anionic surfactants can solubilize positively charged proteins by the electrostatic interaction between the surfactant head groups and the protein surface. Some proteins maintain their catalytic activity in organic media; however, some proteins lose their activity by solubilization into reversed micelles due to the strong protein–surfactant interaction. Cytochrome *c*, which

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is a positively charged protein and takes part in electron transfer reactions, was solubilized in the reversed micelles formulated by di-2-ethylhexyl sulfosuccinate (AOT) with a conformational perturbation. This caused the collapse of its three-dimensional structure [5]. Recently, however, we found that such a structural change of cytochrome *c* by reversed micellar solubilization brought about a catalytic activity as peroxidases in organic media [6]. In our previous work, some phenolic substrates such as pyrogallol and guaiacol were oxidized in the organic solvent by the encapsulated cytochrome *c* in reversed micelles [6]. In the present study, we attempted to oxidize an aromatic amine, *o*-phenylenediamine (*o*-PDA), by cytochrome *c* immobilized in the AOT reversed micellar system. We also discuss here the characteristic performance of cytochrome *c*, which is placed in a nanostructurral environment formed by the molecular assembly.

2. Experimental

2.1. Materials

Horse heart cytochrome *c* was purchased from Sigma and used without further purification. Sodium AOT was obtained from Tokyo Kasei. All other reagents were of highest available purity.

2.2. Experimental methods

A reversed micellar solution containing cytochrome *c* was prepared by direct injection of an aliquot of cytochrome *c* aqueous solution (0.1 M sodium phosphate buffer, pH 8) into a 50 mM AOT/isooctane solution. *o*-Phenylenediamine (*o*-PDA), a hydrogen donor, was directly dissolved in the reversed micellar solution as a substrate. The maximum solubility of *o*-PDA in the AOT reversed micellar solution was approximately 5 mM. The enzyme-catalyzed oxidation was initiated by the addition of a concentrated hydrogen peroxide solution (50 mM AOT, 100 mM H_2O_2, $W_o = 20$) into the reversed micellar solution containing cytochrome *c* and *o*-PDA. Typical reaction conditions are as follows: AOT 50 mM, cytochrome *c* 900 nM, *o*-PDA 5 mM, H_2O_2 0.5 mM, $W_o = 10$, and pH 8.0. The oxidative reaction was detected by a JASCO UVDEC-570 UV–visible spectrophotometer.

3. Results and discussion

Cytochrome *c* is a ubiquitous protein and functions as a part of electron transport systems in vivo; recently some researchers have paid attention to the catalytic performance of cytochrome *c* [7]. In our previous study, cytochrome *c* solubilized in an anionic reversed micellar solution was found to catalyze an oxidative reaction with hydrogen peroxide like peroxidase. In the pyrogallol oxidation, the peroxidase activity of cytochrome *c* solubilized in AOT reversed micelles was found to be 10-fold greater than that in water [6]. Similarly, several organic substrates were oxidized by cytochrome *c* encapsulated in reversed micelles, and provided similar visible spectra based on the typical oxidative products in water: e.g. guaiacol, 2,6-dimethoxyphenol, and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). This function of cytochrome *c* was triggered by a conformational change when solubilized into the anionic reversed micelles through the electrostatic interaction. In particular, many cationic residues are located on the heme crevice side, i.e. the active site of cytochrome *c* for the electron transfer, and thus the active site conformation was readily influenced by solubilization into the reversed micelles. It appears that this conformational perturbation through the electrostatic interaction between the positively charged protein and surfactant headgroups facilitates the accessibility of hydrogen peroxide and organic substrates, and finally provides the peroxidase activity of the solubilized cytochrome *c* in reversed micelles. This mechanism may be similar to the functional conversion of cytochrome *c* by a phosphate bilayer membrane [8,9].

o-Phenylenediamine (*o*-PDA) is also an electron donor for peroxidase. In the present study, we found that cytochrome *c* in reversed micelles can catalyze the oxidative reaction of *o*-PDA. Interestingly, the reverse micellar solution containing the resultant product indicated a blue-colored solution with the absorption peak at 720 nm (Fig. 1A) whereas the

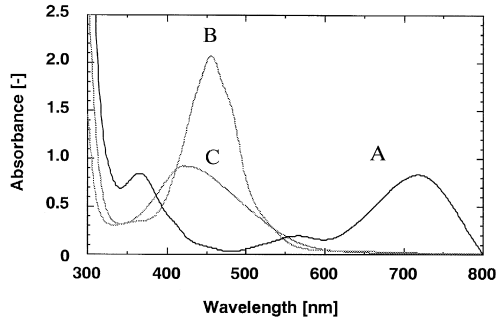


Fig. 1. UV–Vis spectra of the products of the cytochrome *c*-catalyzed oxidation of *o*-PDA in reversed micellar solution, (A: [*o*-PDA] = 2 mM) *o*-PDA·2HCl solubilized in water pool, (B: [*o*-PDA] = 1 mM) and the product of horseradish peroxidase (HRP)-catalyzed oxidation of *o*-PDA solubilized in water (C: [*o*-PDA] = 2 mM).

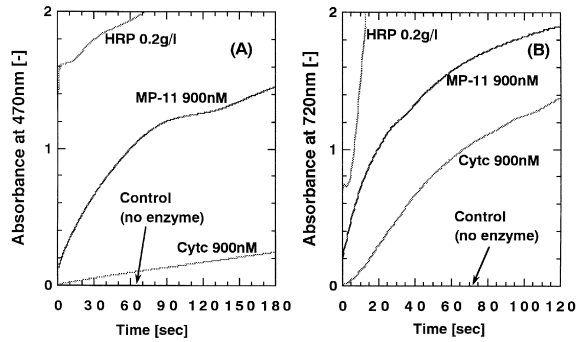
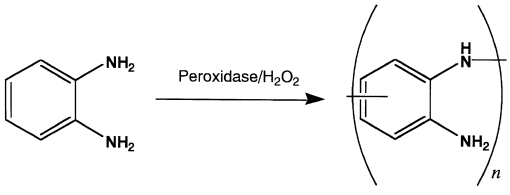


Fig. 2. Time courses of the *o*-PDA oxidation in water (A) and in reversed micellar solution (B) catalyzed by horseradish peroxidase (HRP), micoperoxidase-11 (MP-11), and cytochrome *c* (Cytc). Experimental conditions: AOT 50 mM, *o*-PDA 1 mM, H_2O_2 0.5 mM in isooctane and $W_o = 10$ (pH 8).

oxidative product by horseradish peroxidase (HRP) in water provided a visible spectrum with a maximum absorption at 420 nm, indicating a yellow-colored solution (Fig. 1C). Zhu et al. have suggested that, in water, the oxidative product of *o*-PDA catalyzed by cytochrome *c* was *o*-PDA trimer, 1,2,5,6-tetra-amino-9,10-(1′,2′-di-aminobenzo-)phenanthrene, which has an absorption peak at 470 nm [10]. On the other hand, in an organic solvent Kobayashi et al. reported that the oxidative reaction of *o*-PDA using HRP resulted in polymerization accompanied with the C–N type bond formation as seen in Scheme 1 [11]. These results suggest that the oxidative product catalyzed by cytochrome *c* in the AOT reversed micelles differs from that in water. Fig. 2 shows *o*-PDA oxidation behaviour catalyzed by some heme proteins in water and in a reversed micellar solution. Microperoxidase (MP-11) is a heme peptide, which is obtained by the digestion of cytochrome *c* with proteolytic enzyme, and its catalytic activity as peroxidase has already been supported by several previous reports [12,13]. Fig. 2A clearly exhibited that HRP and MP-11 had higher catalytic activity than that of cytochrome *c*, and all enzymatic products obtained in water showed a typical visible spectrum with the maximum peak at 420 nm. On the other hand, in the reversed micellar solution, the peroxidase activity of cytochrome *c* was considerably enhanced and the oxidative product of *o*-PDA provided a blue-colored solution with a maximum peak at 720 nm as well as that of HRP and MP-11. In addition, from the enhancement of catalytic activity of cytochrome *c* it is clear that reversed micellar solubilization facilitates the functional conversion of cytochrome *c* from an electron transfer protein to a redox enzyme.

Recently Liu et al. [3] prepared the enzymatic-synthesized polyaniline by using HRP in the presence of sulfonated polystyrene (SPS). This water-soluble polyaniline showed specific absorption peaks around 800–1060 and 410–420 nm, which are due to "polaron transitions" based on head-to-tail polymerization, a conducting scheme of polyaniline. They also pointed out that SPS plays an important role in the head-to-tail polymerization of aniline through the electrostatic interaction between SPS and the transitional intermediate of aniline. The resultant product of *o*-PDA obtained in this study also showed two similar peaks in the visible absorption spectrum at 720 and 360 nm, like "polaron transitions". It is considered that a self-assembly of AOT assisted the head-to-tail polymerization as the polyelectrolyte template. In the AOT reversed micellar system, AOT



Scheme 1.

molecules stabilized the reaction intermediate of *o*-PDA by the electrostatic interaction and promoted the head-to-tail polymerization as seen in Fig. 3. Indeed, a blue-colored fraction disappeared in a gel permeation chromatography for the resultant solution (data not shown). This phenomenon can be explained by the dissociation of AOT-oxidative product complex.

When we employed *o*-PDA•2HCl powder as a substrate, which dissolved in water pool of reversed micelles but not in organic solvents, the resultant solution turned yellow (Fig. 1B). This result was similar to the absorption spectrum obtained in water (Fig. 1C). Solubilizing *o*-PDA•2HCl in water pools of reversed micelles leads to the decrease in the pH of the reaction medium including surfactants, cytochrome *c* and *o*-PDA. When 1 mM *o*-PDA•2HCl was dissolved in the reversed micellar solution at $W_o = 10$, the water-pool pH rose to pH 1–2. Consequently, under the *o*-PDA oxidation at low pH a head-to-tail polymerization did not occur because the electrostatic interaction between AOT and the oxidative product of *o*-PDA was prevented from the protonation of a sulfonate group in AOT.

Fig. 4 showed the *o*-PDA oxidation behaviour depending on the inner droplet pH of reversed micelles. With the decrease in pH, *o*-PDA oxidation proceeded more efficiently, whereas the catalytic activity of cytochrome *c* in reversed micelles increased with the increase of the water-pool pH as described in our previous report [6]. At pH 1, however, the product having the maximum absorption at 720 nm was not observed at all as described above. These results suggest that the electrostatic interaction between the resultant product and anionic surfactants play a crucial role in the *o*-PDA oxidation (Fig. 3). Consequently, it was found that the oxidative product containing a head-to-tail structure was induced by the template effect of AOT, but not by the specific oxidation of cytochrome *c*. The isolation and characterization of the oxidative product is now under way.

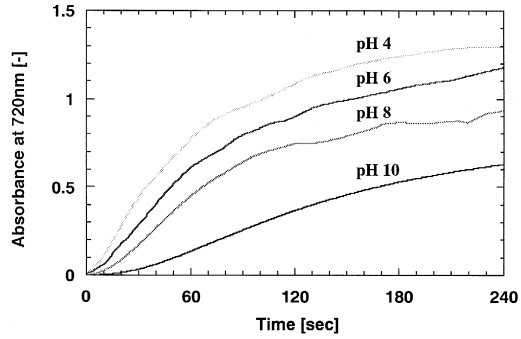


Fig. 4. Effect of the inner droplet pH on the *o*-PDA oxidation in reversed micelles. Experimental conditions: AOT 50 mM, cytochrome *c* 900 nM, *o*-PDA 1 mM, H_2O_2 0.5 mM in isooctane and $W_o = 10$.

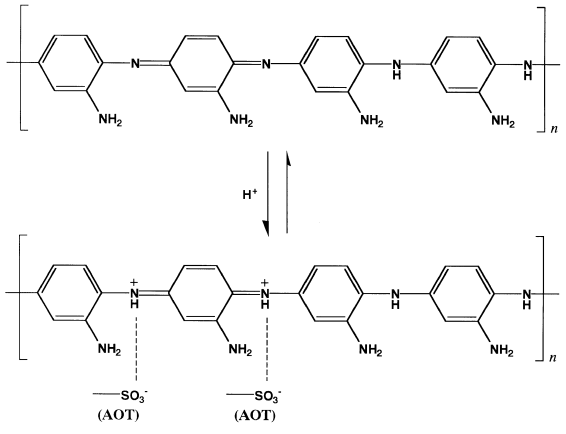


Fig. 3. Schematic illustration of a head-to-tail polymerization of *o*-PDA. The protonated oxidative product was stabilized by the electrostatic interaction with AOT molecules and indicated the polaron transition.

4. Conclusions

The nanostructural environment activated cytochrome *c* entrapped in reversed micelles and facilitated the enzymatic oxidation of *o*-PDA in a nonpolar solvent. The *o*-PDA oxidation catalyzed by cytochrome *c* encapsulated in AOT reversed micelles provided the specific product with an absorption band at 720 nm. This interesting oxidative reaction, however, is not caused by the characteristics of cytochrome *c* because the oxidative products prepared by using different peroxidases, HRP and MP-11, in the AOT reversed micelles also showed similar absorption spectra. Consequently, the AOT reversed micellar medium facilitated an unprecedented *o*-PDA oxidation by the template effect which

stabilized the reaction intermediate of *o*-PDA. Since the resultant product of *o*-PDA is similar to a polyaniline derivative with a head-to-tail structure, an essential structure for polyaniline endowed with electric conductivity, the enzymatic oxidation in reversed micelles possesses a great potential to create a new synthesized polymer.

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