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Structural and redox properties of mitochondrial cytochrome *c* co-sorbed with phosphate on hematite (α-Fe_2O_3) surfaces

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Abstract

The interaction of metalloproteins with oxides has implications not only for bioanalytical systems and biosensors but also in the areas of biomimetic photovoltaic devices, bioremediation, and bacterial metal reduction. Here, we investigate mitochondrial ferricytochrome *c* (Cyt *c*) co-sorption with 0.01 and 0.1 M phosphate on hematite (α-Fe_2O_3) surfaces as a function of pH (2–11). Although Cyt *c* sorption to hematite in the presence of phosphate is consistent with electrostatic attraction, other forces act upon Cyt *c* as well. The occurrence of multilayer adsorption, and our AFM observations, suggest that Cyt *c* aggregates as the pH approaches the Cyt *c* isoelectric point. In solution, methionine coordination of heme Fe occurs only between pH 3 and 7, but in the presence of phosphate this coordination is retained up to pH 10. Electrochemical evidence for the presence of native Cyt *c* occurs down to pH 3 and up to pH 10 in the absence of phosphate, and this range is extended to pH 2 and 11 in the presence of phosphate. Cyt *c* that initially adsorbs to a hematite surface may undergo conformation change and coat the surface with unfolded protein such that subsequently adsorbing protein is more likely to retain the native conformational state. AFM provides evidence for rapid sorption kinetics for Cyt *c* co-sorbed with 0.01 or 0.1 M phosphate. Cyt *c* co-sorbed with 0.01 M phosphate appears to unfold on the surface of hematite while Cyt *c* co-sorbed with 0.1 M phosphate possibly retains native conformation due to aggregation.

Keywords: Adsorption; Phosphate; Cytochrome *c*; Conformation change; Hematite; Oxide

1. Introduction

The interaction of cytochrome *c* with oxide electrodes has been studied for some time [1] because it is important as a baseline for understanding bacterial electron transfer [2], redox catalysis for dehalogenation of halocarbon contaminants [3–5], development of biosensors and bioanalytical systems [6–8], bioremediation [9–13] and emerging photovoltaic applications [14,15]. For new biosensors, direct electrochemistry of redox proteins on electrode surfaces [16] requires that adsorbed proteins retain redox activity. For example, organic ions (4,4′-bipyridine is an early example [17]) have been used to prevent denaturation of adsorbed cytochrome *c* and to orient the heme group toward the surface to promote electron transfer [18]. Boussad et al. [19] showed that phosphate can serve in a similar role for cytochrome *c* sorbed on graphite electrodes. Here, we investigate the co-sorption of mitochondrial cytochrome *c* (Cyt *c*) with phosphate on hematite (α-Fe_2O_3) surfaces, and the effect of phosphate upon Cyt *c* properties while in the sorbed state, using a combination of wet chemical techniques, optical spectroscopy, atomic force microscopy, and cyclic voltammetry. Hematite was chosen because it is the only ferric oxide that occurs, or can be made, sufficiently conductive (through n-type doping) for use as an electrode [20–22].

A study of Cyt *c* co-sorbed with phosphate on hematite surfaces may also have implications for the heterogeneous self assembly of lipids and integral membrane proteins on mineral surfaces, a process thought to possibly have served as a step in the origin of life [23]. In addition, *c*-type cytochromes isolated from the outer membrane of *Shewenella oneidensis* MR-1 (such as OmcA and MtrC) have been implicated in respiratory electron transfer to oxide surfaces [24–27]. Cyt *c*, because it has been studied since the 1920s [28–32] and shares some properties with these outer membrane cytochromes in some conformations (e.g., [33]), makes a useful comparative baseline for work

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with the outer membrane cytochromes. Cyt *c* is a peripheral c-type cytochrome responsible for transferring electrons from the mitochondrial bc_1 protein complex to cytochrome $a-a_3$ complex, and its complexation-induced conformation change allows it to perform its known functions [34]. Because phosphate is relatively common in nature and is known to affect Cyt *c* properties on other surfaces [19], its co-sorption with and effect upon Cyt *c* on hematite is a useful step in understanding the interaction between cytochromes and natural oxide surfaces more generally.

In a previous paper [35], we characterized the adsorption of Cyt *c* on hematite in the absence of phosphate. Our results indicated that sorption of Cyt *c* to hematite is dominated by electrostatic attraction that should orient Cyt *c* molecules with the heme center relatively close to the hematite surface. Direct electrochemistry of Cyt *c* using natural hematite single-crystal electrodes was observed. Although we found no direct evidence of major conformation change upon sorption, the unusually high sorption density, red-shifts in Soret band absorption by Cyt *c* in supernatant solutions, and a slightly negative reduction potential exhibited by Cyt *c* interacting with hematite electrodes as compared to native Cyt *c* are all evidence of possible conformation change upon adsorption. We therefore also studied phosphate co-sorption with Cyt *c* because phosphate may have an influence upon the conformational stability of sorbed Cyt *c*. Our results suggest that Cyt *c* undergoes a conformation change upon interaction with iron oxide, even at pH values for which it remains in the native state in solution.

2. Materials and methods

2.1. Hematite

Hematite was synthesized by the method of Sugimoto et al. [36], and its structure was confirmed using powder XRD. SEM images showed hematite platelets of roughly 1 μm diameter dominated by (001) surfaces. Previous batches of hematite made with this method yielded powders with a N_2 BET surface area 4.76 $m^2\,g^{-1}$. Hematite was washed three times with 1 M KCl solution and further washed with 0.01 M KCl to obtain a 0.01 M KCl background electrolyte [37] and stored as stock aqueous suspension of 94.1 g hematite kg^{-1} (measured) solids concentration.

2.2. Cyt c

Cyt *c* from horse heart was obtained from Sigma and used without further purification. Cyt *c* is a 12.4 kDa globular protein (104 amino acid residues and a covalently attached heme group; PDB ID: 1hrc) with a diameter of about 3.4 nm. Cyt *c* contains 19 lysine residues, of which Lys 13, 27, 72 and 79 are grouped around the heme edge of the molecule. The high lysine content makes Cyt *c* a basic protein, with an isoelectric point of ∼10 [31,38]. The distribution of the lysine residues is not homogeneous, imparting to Cyt *c* dipole moments of 308 and 325 D for the reduced and oxidized forms, respectively [39,40]. This charge distribution is a key factor in the orientation and docking of the cytochrome with cytochrome c oxidase and reductase [41,42] as well as in controlling the redox potentials of the protein (e.g., [41]). The Fe in the heme group is axially coordinated by histidine 18 and methionine 80 in the native state, maintaining the Fe in the low-spin state. The bond between the sulfur of methionine 80 and the Fe of the heme can be disrupted at low and high pH, or at elevated temperature. In some unfolded states, methionine 80 is replaced by histidine 33 [33] to make a his–his coordinated cytochrome, similar to the heme coordination found in cytochromes from iron-reducing and sulfate-reducing bacteria (e.g., [24,42]). The breaking of the Fe–S bond is a mechanism for switching to the high spin state, and allows a portion of the polypeptide chain (from residue 78 to residue 90) to move away from the heme, allowing for ligand replacement. The positively charged edge of Cyt *c* is optimized for electrostatic interaction with negatively charge portions of physiologic partners, and apparently allows interaction with negatively charged electrode surfaces as well. A variety of conformational states have been identified using XANES spectroscopy [43].

2.3. Aqueous experiments

Sorption experiments with hematite suspensions have been described in Khare et al. [35,37]. All samples had a suspended solids concentration of 1.50 g kg^{-1}, constant ionic strength of 0.01 M KCl and total sample mass of 30 ± 0.01 g. Aqueous solutions for sorption experiments (KCl, HCl, KOH all at 0.01 M and Cyt *c* at 0.0001 M) were prepared using analytical grade reagents and degassed (heated and N_2 purged) deionized water. Briefly, 5000 μL of 0.0001 M Cyt *c* solution in 0.01 or 0.1 M phosphate was slowly added to each vigorously stirred sample containing 0.478 g hematite in 0.01 M KCl. The pH was adjusted from 1.7 to 12.3 using a 0.1 M HCl or 0.1 M KOH solution in addition to 0.01 M HCl or 0.01 M KOH for adjusting pH to 1.7, 2.0, or 11.0, 12.0, 12.3, respectively. Each sample was brought to its final mass of 30 g. The sample headspace was flushed with N_2 gas. After equilibration, samples were centrifuged at $\sim 6000g$ for 10 min and the supernatant solutions were decanted and filtered using 0.2-μm polycarbonate membranes. Dissolved Cyt *c* was measured in the supernatant solutions using the Soret band absorption at 408 nm. Sorbed Cyt *c* was determined as the difference between total added Cyt *c* and Cyt *c* measured in supernatants.

2.4. Model calculations

The pH-dependent charge of Cyt *c*, of the surface of hematite in the presence and absence of phosphate, and of Cyt *c* binding to phosphate were calculated as described previously [35] using MICROQL and available surface ionization and phosphate binding constants [44,45]. A Hamaker constant of 10^{-20} J with a simple block model [46,47] was used for approximating van der Waals attraction. Electrostatic forces between a hematite surface and Cyt *c* were estimated using a point charge corresponding to the charge of an Cyt *c* molecule positioned 1.0 nm