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Synthetic, potentiometric and spectroscopic studies of chelation between Fe(III) and 2,5-DHBA supports salicylate-mode of siderophore binding interactions

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ABSTRACT

Catecholate type enterobactin, a prototype siderophore, comprises 2,3-dihydroxybenzoic acid (2,3-DHBA) cyclically linked to serine in *E. coli*. The existence of iron-chelating ligands in humans is a recent discovery, however, the basic chemical interactions between 2,5-dihydroxybenzoic acid and Fe(III) ion remain poorly understood. Achieving an accurate description of the fundamental Fe(III) binding properties of 2,5-DHBA is essential for understanding its role in iron transport mechanisms. Here, we show that 2,5-DHBA binds iron in a salicylate mode via a two-step kinetic mechanism by UV spectroscopy. Complexation between Fe(III) salt and 2,5-DHBA initially occurs at 1:1 ratio (of ligand to metal) and binding resulting in higher-order complexes continues at higher concentrations. Through potentiometric measurements we quantify the distribution of Fe(III)-2,5-DHBA complexes with 1:1, 1:2 and 1:3 stoichiometry. The formation of 1:3 complexes is further supported through high-resolution mass spectrometry. Further, using kinetic and equilibrium UV spectroscopy, we report Fe(III)-2,5-DHBA complex formation at a pH range of 2.5–9.0 at 298.15 K in water. Maximum complexation occurred at a pH range of 4.5–6.5 consistent with deprotonation of the carboxylic acid proton. Equilibrium measurements and stopped-flow kinetics show that complexation rate constants were independent of concentrations of 2,5-DHBA. Together the data supports a model in which the rate-determining step involves rearrangement of ligands on an initial complex formed by reversible binding between the carboxylate group of 2,5-DHBA and Fe(III).

1. Introduction

Recent discoveries reveal the existence of low-molecular weight iron-binding compounds in eukaryotic cells that are capable of mediating iron transportation [1–7]. It was recently reported that 2,5-dihydroxybenzoic acid (gentisic acid) is a key iron-chelating moiety in eukaryotic cells [7]. Indeed, there is a remarkable conservation of the biosynthetic pathways for 2,5-DHBA production in eukaryotes and 2,3-dihydroxybenzoic acid, which is the iron-binding moiety of *E. coli* siderophore enterobactin [8]. In *E. coli*, enterobactin is biosynthesized in a series of reactions catalyzed by at least six enzymes [9]. Among these enzymes, 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (EntA) catalyzes the rate-limiting production of 2,3-dihydroxybenzoate in enterobactin biosynthesis. Significantly, we identified an EntA homologue in eukaryotes, BDH2, another member of the short chain dehydrogenase family responsible for 2,5-DHBA biosynthesis [7]. As with enterobactin, any functional eukaryotic siderophore probably contains additional molecules besides 2,5-DHBA. Nonetheless, 2,5-DHBA alone is sufficient to chelate and transport iron into the mitochondria [7,10].

While the biological role of 2,5-DHBA is an area of intense interest [6,7,11,12], the basic chemical interactions between 2,5-DHBA and Fe(III) ion that are essential for understanding its biological function remain poorly understood. For example, despite evidence for its in vivo function, recent biochemical analyses showed surprisingly low apparent affinity of 2,5-DHBA for Fe(III), and attempts to co-crystallize 2,5-DHBA and Fe(III) bound to lipocalin were unsuccessful [13]. Molecular modeling suggests that the salicylate mode of binding assumed for 2,5-DHBA, in which a carboxyl and an adjacent hydroxyl group on the siderophore interact simultaneously with the bound metal, is incompatible with binding to lipocalin 24p3 [13].

The fact that 2,5-DHBA could effectively transport Fe(III) across the cell in eukaryotes [7] presented an apparent paradox in light of the studies published recently [13] and warranted a reasonable explanation for the binding modes displayed by 2,5-DHBA. Therefore, we examined more closely the complexation reaction of 2,5-DHBA and Fe(III) using

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potentiometric measurements, mass spectrometry, and equilibrium and kinetic UV spectroscopy to characterize the structure and stability of the complexes formed and the mechanism of binding. The data reported here unambiguously demonstrate rapid complexation between Fe(III) salts and 2,5-DHBA through a salicylate mode of binding with multiple 2,5-DHBA ligands interacting with a single Fe(III) metal center. We attribute the appearance of new peaks observed using UV spectroscopy due to formation of Fe(III) complexes, and present further evidence to support this notion through potentiometric analyses and mass spectrometry. The final states of the complexes engage multiple stoichiometry involving 2,5-DHBA as ligands and are likely to involve a 1:3 complex **3**. We also document slow changes in UV absorbance that were consistent with breakdown of the 2,5-DHBA potentially via oxidation involving the hydroxyl group at the 5 position. The long term instability of the complexes observed here would thus explain the inability of previous studies to detect their formation. These data are important in establishing the ability of 2,5-DHBA to complex with Fe(III) ion in vitro and providing a rationale for apparently conflicting biochemical observations regarding 2,5-DHBA complexation properties.

2. Experimental

2.1. Synthesis of Fe(III)-2,5-DHBA complex

2.1.1. Method A

2,5-DHBA (230.0 mg, 1.49 mmol) dissolved in ethanol (4.0 mL) was added to a premixed ethanolic solution of NaOH (60.0 mg, 1.49 mmol in 2.0 mL of EtOH) under constant stirring. Next, $Fe(NO_3)_3 \cdot 9H_2O$ (200.0 mg, 0.50 mmol in 1.0 mL EtOH) was slowly added at room temperature over a period of 20 min. The reaction mixture was vortexed for 30 min to maintain homogeneity of the solution. The complexation over 12 h resulted in precipitation of $NaNO_3$, which was removed by filtration. The resulting solution was evaporated to give a dark blue-colored solid, which was estimated to be 62% of input (158 mg, 0.30 mmol). The solid was analyzed by mass spectrometry after solubilizing in DMSO. The mixture was also analyzed by UV–visible (UV–Vis) spectroscopy after diluting with water.

2.1.2. Method B

2,5-DHBA (342.0 mg, 2.22 mmol) in methanol (10.0 mL) was added to a premixed ethanolic solution of NaOH (89.0 mg, 2.22 mmol in 2.0 mL EtOH) under constant stirring. Next, $FeCl_3 \cdot 6H_2O$ (200.0 mg, 0.74 mmol in 1.0 mL MeOH) was slowly added at room temperature over a period of 30 min. The reaction mixture was stirred for an additional 12 h at room temperature to complete the complexation. Upon complexation, a dark blue-colored mixture was obtained, which was filtered using a Whatman filter. The resulting dark blue-colored complex was estimated to be 66% of input (251.0 mg, 0.49 mmol). The mixture was then analyzed by UV–Vis spectroscopy after diluting with water. The solid was next analyzed by mass spectrometry after solubilizing in DMSO.

2.2. Equilibrium and kinetic UV–Vis spectroscopy of Fe(III) complexes

UV–Vis spectra, in scanning mode, were obtained on a Shimadzu UV-1800 spectrophotometer, which was equipped with a TCC-240A thermoelectrically controlled cell holder (Shimadzu). Data were obtained as absorbance values and were analyzed in Microsoft excel® and Origin®. The time dependent change in absorbance due to chelation was monitored using stopped flow spectroscopy using an Applied Photophysics pi*-180 spectrometer, at fixed wavelength. Solutions of 100–600 μM $FeCl_3 \cdot 6H_2O$ and 2,5-DHBA were mixed using the instruments stopped flow unit with a total shot volume of 150 μL and a flow cell with a 1 cm path length at 298.15 K. The absorbance at 590 nm (2 nm slit width) was collected in 100–400 points over 1–10 s. The rate constants for individual kinetic traces were determined by fitting to a single exponential function. Fits to this equation gave essentially random residuals and averages of >3 individual kinetic traces provided estimates of experimental error of ca. 10%.

2.3. Speciation study using potentiometry

A perchloric acid stock solution was prepared and standardized as described previously [14]. A sodium perchlorate stock solution was prepared and standardized according to Biedermann [15]. Sodium hydroxide titrant solutions were prepared and standardized as described previously [16]. Fe(III) perchlorate was prepared and standardized as reported by Ciavatta et al. [17]. All carbonate free solutions were prepared under inert atmosphere with double distilled water. The cell arrangement was similar to that described by Forsling et al. [18]. Ag/AgCl electrodes were prepared according to Brown [19]. Glass electrodes, manufactured by Metrohm, were of the 6.0133.100 type. They acquired a constant potential within 10 min after the addition of the reagents and remained unchanged within ± 0.1 mV for several hours. The titrations were carried out with a programmable computer controlled data acquisition switch unit 34970 A supplied by Hewlett Packard. The electro motive force (EMF) values were measured with a precision of $\pm 10^{-5}$ V using an OPA 111 low-noise precision DIFET operational amplifier. A slow stream of nitrogen gas was passed through four bottles (a–d) containing: a) 0.96 mol kg^{-1} NaOH, b) 0.94 mol kg^{-1} H_2SO_4, c) twice distilled water, and d) 1.05 mol kg^{-1} $NaClO_4$, and then into the test solutions, stirred during titrations, through the gas inlet tube. During the EMF measurements, the cell assembly was placed in a thermostat kept at (298.15 ± 0.1) K.

2.4. Mass spectrometry

MS analyses were performed on a Thermo Scientific LTQ-FT™ (a hybrid mass spectrometer consisting of a linear ion trap, low resolution) and/or a Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR, TOF-ESMS).

3. Results and discussion

3.1. Synthesis and stability of Fe(III)-2,5-DHBA complex

As shown in Fig. 1, depending on the acid dissociation constant (pK_a), one or multiple protons of 2,5-DHBA may be lost to give rise to a bidentate ligand binding ion (in its salicylate mode) with the possibility of any of the three complexes (**1**, **2** or **3**) as a result of complexation [14]. The pK_a of the three acidic protons in 2,5-DHBA are reported at 3.1 (COOH), 10.46 (C2 phenoxyl) and 13.41 (C5 phenoxyl) respectively [20]. In a simplistic model, without considering stereochemical isomerism, the complexes **1**, **2** and **3** could in principle co-exist at equilibrium, once Fe(III)-salt (e.g. $FeCl_3.6H_2O$ or $Fe(NO_3)_3.9H_2O$) and 2,5-DHBA are complexed in aqueous solution. Through systematic variation of ligand-to-metal stoichiometry, we designed conditions to enable the generation of Fe(III)-2,5-DHBA complexes. The relative populations of each individual complex were expected to be a function of stoichiometry of Fe(III) and 2,5-DHBA. Synthetic methods that were employed for populating the 3:1 complex **3** are shown in Scheme 1. 2,5-DHBA was treated with sodium hydroxide in ethanol as a solvent (in which 2,5-DHBA dissolves completely) to generate the carboxylate ion of 2,5-DHBA. Ferric chloride hexahydrate was pre-dissolved in ethanol and this solution was titrated slowly into the carboxylate salt solution of 2,5-DHBA.

After addition of the aqueous solution of $FeCl_3 \cdot 6H_2O$ to a 2,5-DHBA or 5-MeO-SA solution, a drastic color change was noticed due to the ligand to metal charge transfer and the solution turned from colorless to deep blue instantaneously. When compared to the existing literature on Fe(III) complexes of comparable ligands, the observed color change seems reasonable and expected [21]. After filtration, we obtained a dark blue amorphous solid indicating the formation of