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Single-molecule interaction between a heme protein and carboxylic groups in chemical environments

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Abstract: Noncovalent chemical interactions, including electrostatic interaction, hydrogen-bonding, and hydrophobic interaction, constitute crucial driving forces in the bottom-up assembly of multifunctional molecular complexes and nanomaterials. Electrostatic interaction in chemical environments is generally considered as non-specific interaction, which poses notable challenges for control and direct measurement of electrostatic forces at the single-molecule level. Here, we report a model system consisting of *cytochrome c* and carboxyl group-containing molecule and use electrochemical AFM force spectroscopy to navigate the electrostatic forces of the system under various chemical conditions. Control and effects of solution pH, ion strength, and redox states of *cytochrome c* on electrostatic interactions are systematically studied. This work would provide a useful approach to investigate the intermolecular electrostatic interaction at the single-molecule level.

1. Introduction

Noncovalent chemical interactions, including electrostatic interaction, hydrogen-bonding, and hydrophobic interaction, constitute crucial driving forces in the bottom-up assembly of multifunctional molecular complexes and nanomaterials. Electrostatic interaction is a natural force that exists in almost anything and plays a key role in many natural molecular assemblies. Most of the achievement in surface self-assembled chemistry dependent on electrostatic interaction. All nucleic acids and most biomolecules carry uncompensated electrical charges.^[1] they facility the extreme stability of proteins in thermophilic bacteria,^[2] and increase the strength of many bioadhesives.^[3] Because of their strength and tunability, electrostatic interactions see a growing interest for use in nanotechnology.^[4] Electrostatic interactions can be formed by either ionizable residue side-chains of proteins, or by the free ionized groups of the amino- and carboxy-termini. Electrostatic forces and energies are essential for the interactions of virtually all biological macromolecules^[5]. The central role of electrostatics

is due to the fact that most biological macromolecules, especially DNA and protein, are highly charged. In general, electrostatic interaction is considered as non-specific interaction in a chemical environment, which poses a significant challenge for controlling and directly measuring the electrostatic forces at the single-molecule level.

Atomic Force Microscopy (AFM) has been a powerful tool in biological applications due to its combination of single-molecule resolution, physiological condition, and observation of native substrates.^[6] AFM force spectroscopy has the power to uncover chemically hidden information that would not be revealed by traditional chemical approaches,^[7] for example, mechanical activation of chemical bonds (bond rupture) as well as the influence of stretching forces on chemical reactions.^[8] A large portion of AFM works of interest containing single-molecule force spectroscopy, molecular recognition, and nano-manipulation in physics, chemistry, and biology have been done in air or liquid,^[9] however, electrochemically controlled AFM (ECAFM) has not been applied broadly.^[10] The main reason is due to technical challenges and the use of AFM in liquid environments so far mostly for biochemistry and molecular biology.

To specifically examine the electrostatic interactions at the single-molecule level, one has to carefully consider the effect of the protonation/deprotonation and the environment. Here, we applied ECAFM to directly measure the electrostatic interaction between yeast *cytochrome c* on the substrate and a carboxyl-terminal molecule-tethered AFM tip under different chemical conditions. Yeast *cytochrome c*, which is stable in a broad range of pH, is used as a model system allowing to investigate of the effect of pH, ionic strength, and redox states. We took advantage of ECAFM to quantify the electrostatic interaction at the single-molecule level.

2. Results and Discussion

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2.1 AFM imaging and single-molecule interactions between cytochrome c and a carboxyl group.

To probe the strength of the electrostatic force between single molecules by AFM, we attach a bifunctional group small molecule (16-mercaptohexadecanoic acid) to a gold-coated AFM cantilever (Figure 1c). The small molecule is bound by the thiol-gold bond to the gold-coated AFM tip with its carboxyl-end, which is negatively charged. In control, we designed alkyl terminal hexadecanethiol without charge to attach on gold-coated AFM cantilevers (Figure 1d). Yeast *cytochrome c* is an electron carrier with an iron-containing heme prosthetic group. The iron atom can be either in the oxidized (ferric, Fe^{3+}) or the reduced (ferrous, Fe^{2+}) form. Based on structural specificity, which has a cysteine group and the opposite side have many positive charges, *cytochrome c* can be easily immobilized on the Au(111) substrates by the thiol-gold bond.

Figure 1. Schematic illustration of the experimental setup. (a) AFM imaging and force spectroscopy at non-electrochemical control. (b) AFM imaging and force spectroscopy at electrochemical control. (c) and (d) schematic of *Cytochrome c* interacts with a carboxyl group and alkyl group, respectively. (e) and (f) Typical force-extension curves corresponding to (c) and (d). The black lines represent the trace process as the molecules on the tip approach and interact with *cytochrome c* monolayers. The red lines represent the retracing process as the molecules on the tip withdraw from the monolayer. The blue arrow indicates the electrostatic interaction between *cytochrome c* and a carboxyl group.

Our experimental outline is illustrated in Figure 1 and Figure s1. We then bring the functional cantilever in contact with a substrate on which a *cytochrome c* self-assembly submonolayer, which enriches lots of positive charges (Figure 1a and b). Upon contact, the oppositely charged molecules contribute to electrostatic interaction. We measure the strength of the interaction in aqueous solutions at various chemical conditions. The overall fraction of

force-distance curves in which we find an attractive interaction (blue arrow in Figure 1e) outside the region of contact adhesion. When we repeat experiments with hexadecanethiol modified tips, we only find physical adsorption (Figure 1f).

In order to also demonstrate the formation of *Cytochrome c* submonolayer on Au(111), we applied AFM to image the sample (Figure s2). The AFM images show *cytochrome c* immobilize on Au(111) substrate surface densely in liquid. The apparent height of *Cytochrome c* is about 3.5–4 nm (Figure s2) and consistent with the theory.^[11] In comparison, we carried out images at different pH buffers. The results show that the average height of *Cytochrome c* is slightly different (Figure s2) due to electrostatic interaction between the AFM tip and sample under different pH values.^[12]

2.2 Effects of solution pH on electrostatic forces.

The electrostatic force curve measurements at different pH were recorded by AFM force spectroscopy. The results show that the electrostatic interaction force is about 53 ± 11 pN at pH 4.0, 48 ± 9 pN at pH 7.0, and 33 ± 6 pN at pH 9.7, respectively (Figure 2). From pH 4.0 to 9.7, we can observe the force decrease due to the deprotonation on the surface of *cytochrome c* (Figure s4). *Cytochrome c* contains a large number of basic amino acids (lysine, arginine) and a small number of acidic amino acids. The ability of that the side chains of amino $-\text{NH}_2$ groups bind to protons in solution is much greater than that a small amount of acidic and neutral amino acids lost protons below the isoelectric point. While the total surface charge of *cytochrome c* was closed to neutral state with increasing pH, the interaction was weekend and the electrostatic force decreased.

Figure 2. The electrostatic force in the pH range of 4–11.8. (a) Comparison of typical force-extension curves at pH 4.0, 7.0, 9.7, and 11.8, respectively. (b–d) Histogram of the electrostatic force at pH 4.0, 7.0, and 9.7, respectively. The average electrostatic force decrease with pH increasing. The average forces are 51 ± 17 pN at pH 4.0 ($n = 308$), 48 ± 15 pN at pH 7.0 ($n = 304$) and 33 ± 20 pN at pH 9.7 ($n = 312$), respectively. (e) Comparison of the violin plots of the electrostatic force at different pH, which the forces change significantly with pH.

Further, the force decreases and the probability of interaction reduced significantly at pH 10.3 (Figure s4). When the pH

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exceeds 10.3, there is no interaction between *cytochrome c* and $-\text{COOH}$. The reason is that *cytochrome c* isoelectric point is 10–10.5. When pH is higher than the isoelectric point, there are many negative charges on the surface of *cytochrome c* and the electrostatic attractive force becomes a repulsive force.

2.3 Roles of ionic strength in electrostatic interactions.

While the behaviour at large distances and low salt concentrations is well-described by the standard Poisson–Boltzmann theory, significant deviations due to ion correlations, charge regulation, specific ion effects, hydration occur at high concentration and small separations. As shown in **Figure 3**, we performed the electrostatic force curves measurements in different ionic strength buffer at pH 7 (**Figure 3a**). When the ionic strength is 10 mM, the average electrostatic force is about 50 pN and the average force is about 37 pN at 210 mM concentrate buffer solution (**Figure 3b and Table s1**). The results show that the average electrostatic force decreased significantly with increasing ionic strength. When the salt concentration is 510 mM, the electrostatic interaction of *cytochrome c* and carboxyl group almost disappears (**Figure 3a**), and the possibility of the interaction reduces from about 70% to less than 10% (**Fig.s7**).

Figure 3 Electrostatic force in the solution of salt concentration 10–510 mM. (a) Typical force-extension curves at salt concentration 10 mM, 210 mM, and 510 mM, respectively. (b) The distribution of the electrostatic force between *Cytochrome c* and carboxyl by AFM force spectroscopy at different ionic strengths. (c) The plot of the electrostatic force change with the Debye's length.

To further explain this phenomenon, we estimated the Debye length. In electrolytes, the Debye length is a measure of a charge carrier's net electrostatic effect in a solution and how far its electrostatic effect persists.^[13] At room temperature, the equation can be simplified to estimate the Debye length with the following relation (Eqs (1))^[14]:

$$\kappa^{-1} = 0.304 / \sqrt{I(M)} \quad (1)$$

where κ^{-1} is expressed in nanometers and I is the ionic strength expressed in molar (M or mol/L). The Debye length estimated is plotted with the electrostatic force in **Figure 3c**, which show that the force decrease with the Debye's length reducing. It indicates that the force between *cytochrome c* and carboxyl group conforms to the rule of electrostatic force. Further, the linear

relationship between the electrostatic force and Debye's length is well described by our experimental results as PB theory at low ionic strength (from 10mM to 110mM). However, at high ionic strength, we observe a large deviation from this linear dependence, in agreement with previous reports.^[15]

2.4 Effect of *cytochrome c* redox states on electrostatic forces

Electrochemical AFM is able to control the redox state of transition protein and record force spectra, simultaneously. This ability offers the possibility to investigate how the redox state of metal atom impacts on electrostatic interaction at the single-molecule level. In this study, the heme with an iron-containing compound of the porphyrin is electroactive and its redox state can be controlled by electrochemical potentials applied to the substrate. As our previous record, the formal redox potential is around + (12–50) mV (vs SCE).^[16] A large number of force curves for the electrostatic force at different working potentials were recorded and statistically analyzed by our ECAFM system (**Figure s7**).

Figure 4 The redox-state dependent force between *cytochrome c* and the carboxyl group. (a) Comparison of typical force-extension curves for interactions between *cytochrome c* and carboxyl at different working potentials (vs SCE) of +102 mV (fully oxidized state), -98 mV (partially reduced but close to the equilibrium form), and -298 mV (fully reduced form). The arrows indicate the electrostatic interaction force peaks. The loading rate was 200 nm s⁻¹. (b–d) Corresponding histograms of electrostatic force distributions. The average force based on statistical analyses is: (b) 54 ± 11 pN (n = 341) at +102 mV, (c) 48 ± 9 pN (n = 304) at -98 mV, and (d) 43 ± 7 pN (n = 337) at -298 mV. (e) Comparison of electrostatic force corresponding to (b–d).

Figure 4a compares typical AFM force spectra recorded at +102, -98, and -298 mV, respectively. The corresponding histograms are shown in **Figure 4b–4d**. In the oxidized form (i.e., the working potential applied at +102 mV), the electrostatic force was found to be as high as 120 pN, with an average force of around 54 pN. The force decreased to about 48 pN when the working potential was partially reduced at -98 mV. The force was further weakened to 44 pN at -298 mV where *cytochrome c* was in its fully reduced form. The differences of the electrostatic force at redox states are not large, but significant (**Figure 4e**). The possible reason is that the oxidation state and reduction state of *cytochrome c* have 9 and 8 positive charges, respectively, and the surface charge distribution is uneven. Therefore, when the surface of *cytochrome c* interacts with negatively charged carboxyl groups, the force at oxidized state is slightly higher than at the reduced state due to

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more positive surface charges. Besides, the distribution of forces is narrower for reduced form than oxidized form. We suppose that the positive charge on the surface of *cytochrome c* SAM for the reduced form is less than the oxidized form so that the negative charge of the molecule on the AFM tip is less likely to contact the adjacent positive charge.

Conclusion

To analyze the electrostatic interaction between *cytochrome c* and a carboxyl group, single-molecule force spectroscopy measurements were performed by AFM with controlling. The single-molecule electrostatic force is about 53 pN at pH 4.0 and the force decreases with the increasing of pH from 4.0 to 9.7 due to the deprotonation of *cytochrome c*. When pH exceeds 10.3, there is repulsive interaction between *cytochrome c* and $-\text{COOH}$. The single-molecule electrostatic force and the probability of interaction decrease with the increase of the salt concentration. Under electrochemical control, the electrostatic force changed slightly at different redox states since the difference in the number of surface positive charges between the oxidation state and the reduction state. In summary, we developed a useful approach to investigate the intermolecular electrostatic interaction at the single-molecule level under different chemical conditions.

Experimental Section

Substrate modification. Au(111) substrates were annealed in a hydrogen flame and quenched in Milli-Q water saturated with hydrogen gas. The clean Au(111) was immediately transferred into 10 mM yeast *Cytochrome c* solutions at 4°C for overnight.

In-situ AFM imaging. AFM imaging was performed by 5500AFM (Agilent Technologies, Chandler, AZ). *Cytochrome c* was imaged by AAC mode AFM. Oxide-sharpened Si_3N_4 probes (Bruker, NP-S) with a spring constant of 0.06 N/m were used for imaging the soft biomolecules. The typical radius of NP-S tip is about 10 nm. Imaging amplitude was set between 2.0 and 2.5 V. The scanning speed was about 1.7 Hz. All the images were recorded with 512 × 512 pixels. *Cytochrome c* heights were measured using PicoScan 5.3.3 software (Agilent Technologies, Chandler AZ). The maximum height was taken as the peak height relative to the local background.

Tip modification and force spectroscopy measurements. Silicon-nitride cantilever tips (Bruker, Santa Barbara, CA) for imaging and force spectroscopy measurements. To measure the electrostatic interaction, the tips were coated with gold by plasma sputtering. The thick of the coated layer is 10 nm Cr and 50 nm Au and the typical spring constant is between 0.09–0.11 N/m. The fresh cleaning tips were put in 10 mM $\text{HSC}_{15}\text{H}_{30}\text{COOH}$ or $\text{HSC}_{15}\text{H}_{30}\text{CH}_3$ (99.9%, Aldrich, USA) for overnight at room temperature. The tips were then rinsed in ethanol and stored in a desiccator until use. Force spectroscopy was acquired using an AFM (Agilent Technologies, Chandler, AZ). During force-distance cycles, hundreds of force curves were recorded at different positions of the sample. The force-extension measurements were carried out in two-component buffer with K_2HPO_3 and KH_2PO_3 under pH 4.7, 6.2, 7.0, and 8.5 with a salt concentration of 10 mM. The buffer pH 4, 9.7, 10.3, and 11.8 were adjusted by adding HCl and NaOH, respectively. The different salt concentrations were achieved by adding KCl to the two-component buffer. For each experiment, force-extension measurements were carried out at different pH and salt concentrate using the same cantilever to eliminate errors associated with

cantilever calibration. All experiments were carried out at a pulling speed of 200 nm s⁻¹.

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Keywords: force spectroscopy • single-molecule force • electrostatic interaction • *cytochrome c* • electrochemistry

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