1. Introduction

Electrostatic interactions play a very important role in governing the activity of ions and charged molecules. Therefore, the thermodynamic and kinetic properties of ions and charged molecules are drastically influenced by the ionic strength (I). However, it is difficult to define the $I^*$ value of zwitterions with zero net charge. Nevertheless, zwitterions clearly have charges and seem to show ionic properties. Since the positive and negative charges are close to each other in zwitterions, the electric fields of the charges partially overlap each other and are weakened. Understanding the effect of partially overlapped electric fields within zwitterions on its ionic properties will help us predict the behavior of more complex charged molecules. On the other hand, the electrostatic interactions of proteins are involved in various biological reactions, including binding, molecular recognition, stabilization of intermediates, and enzyme kinetics. Therefore, the understanding of electrostatic interactions is essential for the elucidation of a biological activity. Although electrostatic interaction of proteins may sometimes be discussed in terms of the total net charge, the local charge in the reaction site seems to be more important to understand the electrostatic interaction. The overlapping of the electric fields of charged amino acid residues in close proximity is also important to understand the electrostatic property of proteins. To correctly explain the experimental data, an information on the charge distribution of proteins, i.e., the crystal structure, is required. However, there are only few studies that discussed the experimental data on the electrostatic interactions and crystal structures.1–2

In this study, we attempted to electrochemically detect the electrostatic interaction of charged molecules with partially overlapped electric fields. As the first example, we focused on zwitterions having both positive and negative charged groups with zero net charge. We used a redox couple, $[\text{Fe(CN)}_6]^{3-/4-}$, as the probe ion to measure the electrostatic interaction between the ions and zwitterions via the zwitterion concentration dependence of the formal redox potential ($E^{\circ}$) of $[\text{Fe(CN)}_6]^{3-/4-}$. Linear amino acids with different chain lengths (glycine, $\beta$-alanine, $\gamma$-aminobutyric acid, and $\varepsilon$-aminocaproic acid) were used as zwitterions (Fig. 1A). By analyzing the experimental data, the behavior of the zwitterions in the solution was clarified. For the second example, we used quinohemoprotein amine dehydrogenase (QH-AmDH) from Paracoccus denitrificans and three redox proteins as the electron acceptors: amicyanin, cytochrome $c_{550}$, and horse heart cytochrome c. The second-order reaction rate constant ($k$) of the intermolecular electron transfer between the proteins was electrochemically determined in various ionic strengths ($I$). The $I$ dependences of $k$ were explained not by the net charges but by the local charges around the interaction interfaces of the proteins.

2. Experimental

2.1 Materials

The expression and purification of QH-AmDH,3–5 amicyanin,6–8 and cytochrome $c_{550}$9 from Paracoccus denitrificans were carried out as described in the literature. Horse heart cytochrome c was purchased from Sigma Aldrich and used without further purification. All other chemicals used in this study were of analytical reagent grade, and all solutions were prepared with distilled water.
2.2 Electrochemical methods

The solution potentials were measured with an ADVANTEST R8240 digital electrometer equipped with an ORP electrode (Horiba, 9300-10D). We used glucose and KCl as the non-electrolyte and strong electrolyte, respectively. For zwitterions, we used glycine (H\textsubscript{2}N\textsuperscript{+}CH\textsubscript{2}COO\textsuperscript{-}, \(pK_a\) (NH\textsubscript{3}\textsuperscript{+}) = 9.91, \(pK_a\) (COOH) = 2.36), \(\beta\)-alanine (H\textsubscript{2}N\textsuperscript{+}CH\textsubscript{2}CH\textsubscript{2}COO\textsuperscript{-}, \(pK_a\) (NH\textsubscript{3}\textsuperscript{+}) = 10.2, \(pK_a\) (COOH) = 3.55), \(\gamma\)-aminobutyric acid (H\textsubscript{2}N\textsuperscript{+}CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}COO\textsuperscript{-}, \(pK_a\) (NH\textsubscript{3}\textsuperscript{+}) = 10.4, \(pK_a\) (COOH) = 4.23), and \(\varepsilon\)-aminocaproic acid (H\textsubscript{2}N\textsuperscript{+}CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}COO\textsuperscript{-}, \(pK_a\) (NH\textsubscript{3}\textsuperscript{+}) = 10.7, \(pK_a\) (COOH) = 4.43). Based on the \(pK_a\) values, these amino acids may exist in zwitterion form when dissolved in pure water. The measurements were carried out in a solution containing 0.1 mM \([\text{Fe(CN)}\textsubscript{6}]^{3-}\) and 0.1 mM K\textsubscript{4}[Fe(CN)\textsubscript{6}] in several concentrations of zwitterion solutes. All solutions (except KCl solutions) contained KCl with a final concentration of 5 mM as the supporting electrolyte. The temperature of the measurement solutions was maintained at 25 ± 1°C using a water-jacketed cell with a solution volume of 2.0 mL.

The \(E^{\prime}\) value of the \([\text{Fe(CN)}\textsubscript{6}]^{3-/4-}\) couple was defined as the standard redox potential of \([\text{Fe(CN)}\textsubscript{6}]^{3-/4-}\) to which the standard electrode, \(R\), is the gas constant, \(T\) is the absolute temperature, \(F\) is the Faraday constant, and \(a\) is the activity of ion \(i\). Rewriting with the activity coefficient (\(\gamma_i\)) and the molar concentration of ion \(i\) (\(c_i\)), Eq. (1) becomes

\[
E = E^o + \frac{RT}{F} \ln \frac{\gamma_i [	ext{Fe(CN)}\textsubscript{6}]^{3-}}{\gamma_i [	ext{Fe(CN)}\textsubscript{6}]^{4-}} (1)
\]

where \(E^o\) is the solution potential, \(E^o\) is the standard redox potential of \([\text{Fe(CN)}\textsubscript{6}]^{3-/4-}\), \(R\) is the gas constant, \(T\) is the absolute temperature, \(F\) is the Faraday constant, and \(a\) is the activity of ion \(i\). Rewriting with the activity coefficient (\(\gamma_i\)) and the molar concentration of ion \(i\) (\(c_i\)), Eq. (1) becomes

\[
E = E^o + RT \ln \frac{\gamma_i [	ext{Fe(CN)}\textsubscript{6}]^{3-}}{\gamma_i [	ext{Fe(CN)}\textsubscript{6}]^{4-}} (1)
\]

where \(E^o\) is the standard redox potential, \(E^o\) is the standard redox potential of \([\text{Fe(CN)}\textsubscript{6}]^{3-/4-}\), \(R\) is the gas constant, \(T\) is the absolute temperature, \(F\) is the Faraday constant, and \(a\) is the activity of ion \(i\). Rewriting with the activity coefficient (\(\gamma_i\)) and the molar concentration of ion \(i\) (\(c_i\)), Eq. (1) becomes

\[
E = E^o + RT \ln \frac{\gamma_i [	ext{Fe(CN)}\textsubscript{6}]^{3-}}{\gamma_i [	ext{Fe(CN)}\textsubscript{6}]^{4-}} (1)
\]

with

\[
\frac{E^{\prime}}{E} = \frac{RT}{F} \ln \frac{\gamma_i [	ext{Fe(CN)}\textsubscript{6}]^{3-}}{\gamma_i [	ext{Fe(CN)}\textsubscript{6}]^{4-}} (3)
\]

From Eq. (2), \(E^{\prime}\) equals \(E^o\) under our conditions (\(c_{[\text{Fe(CN)}\textsubscript{6}]^{3-}} = c_{[\text{Fe(CN)}\textsubscript{6}]^{4-}}\)). Since \(\gamma_i\) is strongly influenced by the electrostatic interaction between ion \(i\) and other electrolytes, \(E^{\prime}\) reflects the electrostatic interaction.

Cyclic voltammetry was performed on an HZ-3000 (Hokuto Denko Co.). Au electrodes (diameter = 1.6 mm) and thiol-modified Au electrodes were used as the working electrodes (BAS, Inc.). The bare Au electrode was polished to a mirror-like finish with Al\textsubscript{2}O\textsubscript{3} powder (particle size = 0.05 μm), rinsed with distilled water, and sonicated in distilled water. The thiol-modified Au electrodes (Fig. S1) were prepared by immersing the polished Au electrodes in a saturated solution of 4,4’-dipyridyl disulfide for more than 30 min; they were washed thoroughly with distilled water before use. A Pt wire and Ag/AgCl sat. KCl electrode were used as the counter and reference electrodes, respectively. All potentials were referred to the Ag/AgCl sat. KCl reference electrode. The measurements were carried out in 10 mM potassium phosphate buffer with \(pH = 7.5\). The \(I\) value was adjusted to a given value with KCl.

The measurement solution contained 20 mM \(n\)-butylamine as the electron donor, and measurements were performed under Ar-saturated conditions at 25 ± 1°C using a water-jacketed cell with a solution volume of 1.0 mL. Under the conditions of the limiting current without the substrate concentration polarization, that is, when the concentration of the first substrate (\(n\)-butylamine in this case) is sufficiently larger than the Michaelis constant for the substrate and the electrode potential is more positive than the formal redox potential of the mediator (amicyanin: 34 mV, cytochrome \(c_{550}\): 44 mV, and horse heart cytochrome \(c\): 54 mV vs. Ag/AgCl at \(pH = 7.5\)), the limiting catalytic current \(i_{lim}\) is given by

\[
i_{lim} = FA \sqrt{2n_{e}D_{M}k_{cat}K_{M}V_{C}} \left( \frac{CM}{KM} - \ln \left( 1 + \frac{CM}{KM} \right) \right) (4)
\]

where \(A\) is the surface area of the electrode, and \(k_{cat}\) is the catalytic rate constant. \(n_{e} = 1\) in this case, \(D_{M}, k_{cat},\) and \(c_{th}\) are the number of electrons, diffusion constant, Michaelis constant, and the bulk concentration of the electron acceptor protein that works as the mediator in mediated bioelectrocatalysis. \(n_{s} = 2\) in this case is the number of electrons of \(n\)-butylamine as the substrate, and \(c_{th}\) is the bulk concentration of QH-AmDH as the enzyme. From the nonlinear regression analysis of \(i_{lim}\) vs. \(c_{th}\), \(k_{cat}\) and \(K_{M}\) were evaluated, and then \(k_{cat}/K_{M}\) was obtained.

3. Results and Discussion

3.1 The electrostatic interaction of zwitterions

Figure 2 shows the \(E^{\prime}\) of \([\text{Fe(CN)}\textsubscript{6}]^{3-/4-}\) in various solution concentrations. When non-electrolyte glucose solutions were used, \(E^{\prime}\) was independent of the glucose concentration (Fig. 2A). This indicates that no electrostatic interaction occurs between \([\text{Fe(CN)}\textsubscript{6}]^{3-/4-}\) and glucose. When strong electrolyte KCl solutions...
were used, $E''$ shifted to the positive direction with an increase in KCl concentration (Fig. 2B). The charges of the probe ions ([Fe(CN)$_6$]$^{3-}$) are electrostatically compensated by the atmospheric ions (K$^+$ and Cl$^-$) and the prove ions are stabilized by $-RT\ln \gamma$ in the chemical potential. The result of Fig. 2B means that $\gamma_{[Fe(CN)_{6}]^{3-}}/\gamma_{[Fe(CN)_{6}]^{4-}}$ increases with an increase in the KCl concentration as judged with Eq. (3), and then indicates that the electrostatic compensation of [Fe(CN)$_6$]$^{3-}$ is stronger than that of [Fe(CN)$_6$]$^{4-}$ because of its large negative charge.

Based on the Debye–Hückel limiting law, the activity coefficient of ion $i$ is given by:

$$\log \gamma_i = -A_i^{1/2} \frac{z_i^2 I}{1}$$

where $A$ is the constant at a given temperature, and $z_i$ is the charge number of ion $i$. Introducing Eq. (5) into Eq. (3) gives

$$E'' = E^0 + \frac{2.303RT}{F} \log \left( \frac{\gamma_{[Fe(CN)_{6}]^{3-}}}{\gamma_{[Fe(CN)_{6}]^{4-}}} \right)$$

$$= E^0 + \frac{2.303RT}{F} A^{1/2} (-z_{[Fe(CN)_{6}]^{3-}}^2 + \frac{z_{[Fe(CN)_{6}]^{4-}}}{z_{[Fe(CN)_{6}]^{3-}}^2})$$

$$= E^0 + \frac{2.303 \times 8.3145 \times 298.15}{96485} \times 0.5091^{1/2}$$

$$= E^0 + 0.211^{1/2} (\text{at } T = 298.15 \text{ K})$$

where 0.5091 is the value of $A$ in water solution at 298.15 K. The $I^{1/2}$ dependence of $E''$ of [Fe(CN)$_6$]$^{3-}$ is shown in the inset of Fig. 2B. Here the ionic strength contribution from the probe ions (0.1 mM K$_3$[Fe(CN)$_6$] and 0.1 mM K$_4$[Fe(CN)$_6$]) was also taken into account in the calculation of $I$.
agree with the analytical formula (Eq. (6)); this indicates a typical ion-ion electrostatic interaction described by the Debye–Hückel limiting law, in which the atmospheric ions are in a Boltzmann distribution around the center ion. Therefore, the \( E' \) of [Fe(CN)\(_6\)]\(^{3-/+} \) was a good measure in the assessment of the electrostatic properties of solutes. On the other hand, the experimental values deviated from those expected by Eq. (6) in high \( I \) region, because the Debye–Hückel limiting law does not hold at high \( I \).

In the case of zwitterions, \( E' \) also shifted to the positive direction with an increase in the concentration of all zwitterions (Figs. 2C, 2D, 2E, and 2F). However, the degrees of the changes were smaller than KCl solutions. The result clearly indicates that zwitterions show electrostatic properties despite the zero net charge. However, the electrostatic interactions were weaker than that of strong electrolytes, such as KCl. With an increase in the number of the methylene group \((\text{CH}_2)\), the degree of the \( E' \) shift increased. This tendency was coincident with an intuitive interpretation that when the charges in a zwitterion get far apart, it may behave like a strong electrolyte since the overlap in electric fields is reduced.

To analyze the data, we postulate that a zwitterion is a sphere housing dipole charges \((+\overset{\circ}{z} w \epsilon \) and \( -\overset{\circ}{z} w \epsilon \), \( \epsilon \) being the elementary charge) at a distance \( (d) \). The high-order electrostatic interactions (such as dipole–dipole and quadrupole–quadrupole interactions) were ignored. Under these assumptions, the analytical formula of the activity coefficient of ion \( i \) in the presence of a zwitterion is given by Kirkwood.\(^{21} \)

\[
\log \gamma_i = -\frac{3N_Ae^2}{2.303 \cdot 32\pi \epsilon_0 \epsilon_r k_B T^2 a} \cdot \frac{1 - \frac{\kappa a}{2} + \frac{k^2 a^2(1 + \kappa a)}{6}}{1 + \kappa a + \frac{k^2 a^2}{3}} e^2 \xi_{zw} + \frac{e^2 \xi_{zw}}{2}
\]

(7)

with

\[
\kappa = \left(\frac{2N_Ae^2}{\epsilon_0 \epsilon_r k_B T}\right)^{1/2}
\]

(8)

\[
p = z_{zw} \epsilon_d
\]

(9)

where \( N_A \) is the Avogadro’s number, \( \epsilon_0 \) is the permittivity of the vacuum, \( \epsilon_r \) is the relative permittivity, \( k_B \) is the Boltzmann constant, \( a \) is the minimum distance between ion \( i \) and a zwitterion, \( \kappa \) is the reciprocal of the Debye length, \( p \) is the dipole moment, and \( \xi_{zw} \) is the molar concentration of a zwitterion. Equation (7) means that the electric field generated by the center ion \( i \) induces a given biased dipole orientation, and the degree of the biased dipole orientation depends on the magnitude of the electric field and \( p \). Substituting Eq. (7) into Eq. (3) gives

\[ E' = E' + \frac{2.303RT}{F} \log \left( \frac{\gamma_{[\text{Fe(CN)}_6]^{3-}+}}{\gamma_{[\text{Fe(CN)}_6]^{3-}}(a^+)} \right) \]

(10)

Here, we attempted to fit Eq. (10) to the experimental data of the zwitterion solutions by using \( d \) as the fitting parameter. For the value of \( a \), we used the sum of the radius of the crystal structure of [Fe(CN)\(_6\)]\(^{3-/+} \) (= 3.1 Å)\(^{22} \) and half of the roughly estimated length of the linear configuration of zwitterions (Table 1). The fitting results are shown as straight lines in the insets of Figs. 2C, 2D, 2E, and 2F, and the refined data are summarized in Table 1. For glycine, we obtained \( d = 3.3 \) Å. When a linear configuration for glycine was assumed, the distance between the amino group and carboxyl group was 3.6 Å. The structure-based estimated value was consistent with the \( d \) value obtained by the fitting. For \( \beta \)-alanine, \( \gamma \)-aminobutyric acid, and \( \epsilon \)-aminocaproic acid, the \( d \) value increased with an increase in the number of CH\(_2\); \( d = 3.7 \) Å, 4.3 Å, and 4.8 Å, respectively. The \( d \) values obtained by the fitting were smaller than the structure-based estimated values for the linear configuration (4.8 Å, 6.1 Å, and 7.3 Å, respectively). The degree of freedom of the C–C bond of these zwitterions was high, and the positive and negative charges may be located near each other in a bent shape in solutions. In the case of zwitterions with longer alkyl chains, the charges within the zwitterions may behave as independent ones, just like K\(^+\) and Cl\(^-\). However, when the alkyl chain length increases, the effect of the molecular size becomes larger than that of the electrostatic interaction. Therefore, we expect that the change in \( E' \) of zwitterions with sufficiently long alkyl chains will be smaller than that of KCl.

3.2 The electrostatic interaction between QH-AmDH and the electron accepter proteins

Steady-state bioelectrocatalytic waves were observed in cyclic voltammetry of \( n \)-butylamine solution (20 mM) containing QH-AmDH and any of the electron accepter proteins (amineyanin,
cytochrome $c_{550}$ and horse heart cytochrome $c$) at the 4,4′-dipyridyl disulfide-modified Au electrode (Fig. S2). Such catalytic voltamograms were recorded in various values of $I$, which was adjusted with KCl. From the dependence of the steady-state limiting catalytic currents ($\bar{i}_{\text{lim}}$) on the concentration of the electron acceptor protein ($c_{\text{fA}}$), the second-order reaction rate constant ($k$) between QH-AmDH and electron acceptor was evaluated based on Eq. (4).

In the cases of amicyanin and cytochrome $c_{550}$ as the electron acceptors, $k$ decreased with an increase in $I$. The results indicate the electrostatic attractive interaction between the proteins, and the interactive force decreased with an increase in $I$. Such situations should occur when QH-AmDH and electron acceptor proteins are oppositely charged. However, QH-AmDH, amicyanin, and cytochrome $c_{550}$ are all negatively charged as a whole at pH 7.5. As described in section 3.1, zwitterions with zero net charge show electrostatic properties, but the electrostatic interaction is weakened by the overlapping (or compensating) electric fields of oppositely charged sites. Proteins are multivalent polymeric ions with several local charges from its acidic or basic amino acid residues. However, the distances between the charged amino acid residues are often very short, and the electric field of the charged residues must be often weakened in the proteins. Therefore, we should focus not on the net charges but on the local charges of the interacting surface to understand the electrostatic interaction of proteins.

In order to evaluate the local charges, we calculated the electrostatic potential maps of the proteins based on the crystal structure data. Figure 4 shows the electrostatic maps of the proteins examined in this study. At pH 7.5, the net charge of QH-AmDH was calculated to be −46e, and the interface that was anticipated to face with the electron acceptors showed negative potentials. Although the net charges of amicyanin and cytochrome $c_{550}$ were calculated to be −3e, the reaction interface around the redox centers (Cu and heme $c$, respectively) showed positive potentials. These situations of the local charges around the reaction interface agreed with the experimental results, indicating that the local charge distribution of proteins is important to understand their electrostatic interaction.

However, when horse heart cytochrome $c$ was used as the electron acceptor, $k$ increased with $I$. The result suggests that QH-AmDH and horse heart cytochrome $c$ carry the same charges at the reaction interfaces. The electrostatic repulsion was weakened by an increase in $I$. Based on the crystal structure, the net charge of horse heart cytochrome $c$ was calculated to be +8e, and a temporarily assigned interface around the redox center (heme $c$) showed positive potentials at pH 7.5 (Fig. 4D upper image). Since the reaction interface near the redox center of QH-AmDH was negatively charged, the true reaction interface of horse heart cytochrome $c$ was considered to have a negative charge based on the experimental results. Fortunately, the opposite surface from the redox center showed negative potentials (Fig. 4D lower image), and this surface was considered to be the reaction interface with the negatively charged interface of QH-AmDH. Since horse heart cytochrome $c$ is not a physiological electron acceptor of QH-AmDH, the opposite surface of the redox center of horse heart cytochrome $c$ probably interacts with QH-AmDH. The fact that the $k$ value between QH-AmDH and horse heart cytochrome $c$ is the largest in the three protein mediators seems to support the prediction that the interaction interface of horse heart cytochrome $c$ is different from the normal one.

4. Conclusion

To understand the electrostatic interactions between ions (or molecules) with oppositely charged sites, we conducted thermodynamic and kinetic experiments using electrochemical techniques for structurally identified zwitterions, QH-AmDH, and electron acceptors. The shift in the $E^\circ$ of [Fe(CN)₆]⁢−⁴/⁢− is a good measure of the electrostatic interaction of ionic solutions because of the large charge numbers of ions. The effects of the concentration of zwitterions with zero net charge on $E^\circ$ of [Fe(CN)₆]⁢−⁴/⁢− showed weak but clear electrostatic property. The partial overlapping of the electric field of oppositely charged sites weakens the electrostatic interaction of zwitterions. Linear zwitterions with high degrees of freedom in the C–C bonds between two oppositely charged sites appear to show a bent shape in solutions.

We also studied the electrostatic interaction of proteins that contain various charged amino acid residues in close proximity via the $I$ dependence of the second-order reaction rate constant ($k$) in the

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**Figure 3.** $I$ dependence of $\log k$ values between QH-AmDH and various electron acceptor proteins: open circle = amicyanin, closed triangle = cytochrome $c_{550}$, and closed square = horse heart cytochrome $c$.

**Figure 4.** Electrostatic potential maps of (A) QH-AmDH, (B) amicyanin, (C) cytochrome $c_{550}$, and (D) horse heart cytochrome $c$. The circle shows the estimated interaction interface of QH-AmDH. The color transition from red to white to blue represents the electrostatic potential values of $−5kT/ε$ to 0 to $5kT/ε$. To calculate the charges of each atom subsequent to energy minimization, missing hydrogens, atomic radii, and partial charges were added to the coordinates of the structure using PDB2PQR version 2.0.24 with PARSE force field at pH 7.5, thus converting the PDB files to PQR files. In all calculations, water molecules were removed beforehand, and the charges of metal atoms were ignored. Using the PQR files, electrostatic potentials were calculated by APBS version 1.4.25 Electrolyte ions were monovalent, and the concentration of the cation and anion was set to 0.15 M. The radii of the solvent and electrolyte ions were set to 1.4 Å and 2 Å, respectively. Surface potentials are colored according to the potentials of the solvent accessible surfaces.
intermolecular electron transfer. It was revealed that the local charges, not the net charge, play an important role in the electrostatic protein–protein interactions. Electrostatic attractive forces are involved in the complex formation between QH-AmDH and amicyanin or cytochrome c550. The result is explained in terms of the local charges in interacting interfaces; QH-AmDH has some negative charges on the interface near the outer heme c, and some positive charges are located on the interface around the redox center of amicyanin and cytochrome c550. On the other hand, the electrostatic repulsive force is involved in the interaction between QH-AmDH and horse heart cytochrome c. Considering the results, together with the crystal structural data, the interaction interface of horse heart cytochrome c estimated from the analysis may be located on the opposite side of the interface around the redox center. This study could be a good basis on how to evaluate and understand the electrostatic interaction of charged groups of zwitterions and multivalent proteins. We believe that it will lead to a better understanding of biological reactions involving electrostatic interactions.

Supporting Information

The Supporting Information is available on the website at DOI: https://doi.org/10.5796/electrochemistry.21-00030.

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