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THE OXIDATION OF FERROCYTOCHROME *c* BY Br_2^-, $(SCN)_2^-$, N_3 AND OH RADICALS STUDIED BY PULSED-ELECTRON AND γ-RAY RADIOLYSIS

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Summary

The reactions of ferrocytochrome *c* with Br_2^-, $(SCN)_2^-$, N_3 and OH radicals were followed by measuring the change in the optical spectra of cytochrome *c* on γ-irradiation as well as the rate of change of absorbance upon pulse irradiation.

Ferrocytochrome *c* is oxidized to ferricytochrome *c* by Br_2^-, $(SCN)_2^-$ or N_3 radical with an efficiency of about 100% through a second-order process in which no intermediates were observed. The rate constants in neutral solutions at $I = 0.073$ are $9.7 \cdot 10^8\ M^{-1} \cdot s^{-1}$, $7.9 \cdot 10^8\ M^{-1} \cdot s^{-1}$, $1.3 \cdot 10^9\ M^{-1} \cdot s^{-1}$ for the oxidation by Br_2^-, $(SCN)_2^-$ and N_3 radicals, respectively. The rate constants do not vary appreciably in alkaline solutions (pH 8.9). The ionic strength dependence was observed for the rate constants of the oxidation by Br_2^- and $(SCN)_2^-$. Those rate constants estimated on the assumption that the radicals react only with the amino acid residues with the charateristic steric correction factors were less than one-tenth of the observed ones. These results suggest that the partially exposed region of the heme is the probable site of electron transfer from ferrocytochrome *c* to the radical.

Hydroxyl radicals also oxidize ferrocytochrome *c* with a high rate constant ($k > 1 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$), but with a very small efficiency (5%).

Introduction

The reactions of cytochrome *c* with a wide variety of redox agents have been investigated by various techniques [1,2]. Kinetic and thermodynamic studies

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Abbreviations: cyt-III, ferricytochrome *c*; cyt-II, ferrocytochrome *c*.

on the reactions of cytochrome *c* with many well-characterized nonphysiological redox agents are expected to reveal the mechanisms of electron transfer which might be operative in enzymatic systems. Although a few types of mechanism for the redox reactions have been proposed, no evidence for them has been provided without ambiguity. Cytochrome *c* is unlikely to be easily reduced or oxidized by the axial path that requires substitution in the inner coordination sphere because the heme iron is buried deeply inside the protein.

A pulse-radiolysis technique is known to be convenient for studying the reactions of free radicals. This technique has been applied to the reduction of ferricytochrome *c* by e^-_{aq} [2], H [2,3], O_2^- [4—7], CO_2^- [2,3] and a variety of organic free radicals [2,3,8]. The oxidation of ferrocytochrome *c* by nonphysiological oxidants, however, has been less extensively examined than its reduction.

Since radical anions, X_2^-, formed secondarily upon radiolysis of aqueous solutions of halide and pseudohalide ions, react selectively with amino acids [9], they have been widely used to probe functional amino acid residues of various enzymes [10,11]. The oxidation of tryptophan and tyrosine by X_2^- involves mainly one-electron transfer [12—14]. Azide radicals, N_3, were also found to react by fast one-electron oxidation with tryptophan [15]. In a recent study on the reaction of alcohol dehydrogenase with N_3 Land and Prütz [16] reported a long-range intramolecular charge transfer between tyrosine and tryptophan. The reactions of hemeproteins with these selective free radicals (X_2^- and N_3), however, have not been reported. These free radicals may have certain advantages, because of their selectivity, over known oxidizing agents in elucidating the reaction mechanisms of metalloproteins.

In the present study it was demonstrated that cytochrome *c* is oxidized from the ferrous to ferric state by these free radicals (X_2^- and N_3). The efficiency of the conversion and the kinetic data for the reactions were also measured to gain insight into the site on the heme protein at which electron transfer takes place.

Experimental

Materials and preparation of solutions

Horse-heart ferricytochrome *c* (cyt-III) was purchased from Sigma (type III). Ferrocytochrome *c* (cyt-II) was prepared by reducing cyt-III with an approximately 5-fold excess of sodium ascorbate and removing the excess reducing agent on Sephadex G-25. Triply distilled water was used. Other reagents were of analytical grade and were used without further purification.

Cyt-II solutions were prepared less than 15 h before use and stored at 5°C. The concentration of cyt-II was determined from the absorption at 550 nm, using a molar extinction coefficient of $2.77 \cdot 10^4\ M^{-1} \cdot s^{-1}$ [17]. The pH of the solutions was adjusted by adding 2 mM phosphate buffer (for pH 7) or by adding NaOH (for pH 9).

Apparatus and procedures

A Van de Graaff type electron-accelerator at the Institute of Physical and Chemical Research delivered 100 mA electron pulses of 2.6 MeV with durations of 0.5—2 μs. Analyzing light passing once through the solution was conducted to a Baush and Romb monochromator and then monitored by a Hamamatsu-TV photomultiplier (R 374). The optical path length was 1 or 2 cm.

The solutions, which were contained in a 200 ml glass vessel, were purged with N_2O for at least 20 min to remove air. The N_2O-saturated solutions were transferred into the irradition cell (2—4 ml in volume) through a capillary tube. Only one pulse was delivered to a given aliquot of the solution. The absorbed dose per pulse was determined from the transient $(SCN)_2^-$ absorption formed upon pulse radiolysis of air-saturated aqueous solutions of 10^{-2} M NaSCN, using $G((SCN)_2^-) = 2.9$ and $\epsilon_{500}((SCN)_2^-) = 7100 \cdot M^{-1} \cdot cm^{-1}$ [18]. Absorbed doses were controlled within the range from 0.3 to 1.0 krad per pulse (equivalent to $1.8 \cdot 10^{-6}$—$6 \cdot 10^{-6}$ M of reactant radicals) to ensure that the concentration of reactant radicals were approximately one-tenth of that of cyt-II. Absorption spectra of γ-irradiated solutions were recorded on a Cary 14R spectrophotometer. All experiments were carried out at ambient temperature (22 ± 2°C) unless otherwise noted.

Radiolysis of aqueous solutions produces e_{aq}^-, H and OH as primary reactive species. In the presence of Br^-, SCN^- or N_3^-, OH radicals react rapidly with either of them to produce free radicals (X). With the exception of N_3^-, which gives azide radicals (N_3) [19], the radicals (X) form dimer radical anions (X_2^-) [20,21]. In the N_2O-saturated solutions, e_{aq}^- is converted to OH. Then, Br_2^-, $(SCN)_2^-$ or N_3 radicals are formed with the same yield as the combined yield of e_{aq}^- and OH: $G \approx 5.8$ molecules/100 eV.

$$e_{aq}^- + N_2O \rightarrow N_2 + OH + OH^- \quad k = (8.67 \pm 0.6) \cdot 10^9 \, M^{-1} \cdot s^{-1} \quad [22]$$

$$X^- + OH \rightarrow X + OH^- \quad k \approx 10^9 - 10^{10} \, M^{-1} \cdot s^{-1} \quad [23]$$

$$X + X^- \rightleftharpoons X_2^-$$

Even when cyt-II is present as an additional reactant, reactions of cyt-II with e_{aq}^- or OH ($k = 1 \cdot 10^{10}$—$5 \cdot 10^{10} \, M^{-1} \cdot s^{-1}$ [2,24]) are neglected if appropriate concentrations of the reactants are taken: in the N_2O-saturated solutions ($[N_2O] = 25$ mM) containing X^- (0.1 M) and cyt-II ($1 \cdot 10^{-5}$—$3 \cdot 10^{-5}$ M), more than 99% of e_{aq}^- was scavenged by N_2O and more than 95% OH react with X^-. The yield of H is only about 10% of the total yield of e_{aq}^- and OH. Therefore, under the above experimental conditions either Br_2^- or N_3 can be regarded as the only reagent capable of reacting with cyt-II, and OH is the only reactant when X^- is not present.

Results

The reactions of cyt-II with Br_2^-, $(SCN)_2^-$, N_3 and OH radicals were followed by measurement of the optical absorption spectra after γ-irradiation. The spectrum of cyt-II changed into that of cyt-III with the isosbestic points reported [17]. From the spectral changes it was found that cyt-II was oxidized to cyt-III by the reaction with Br_2^-, $(SCN)_2^-$, or N_3 until all cyt-II was converted to cyt-III.

Plots of the amounts of cyt-II oxidized by these radicals against dose are shown in Fig. 1. A good linear relation is obtained below 8 krad for each radical. The G values for the oxidation by Br_2^-, $(SCN)_2^-$, N_3 and OH were determined

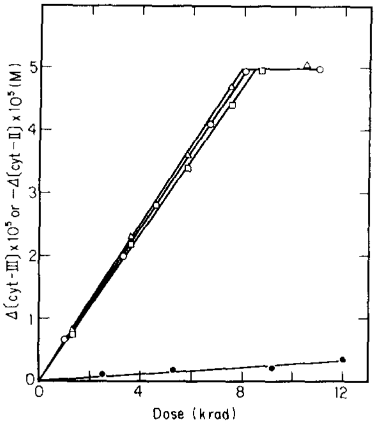


Fig. 1. The amounts of cytochrome *c* converted by the reactions with Br_2^- (○), $(SCN)_2^-$ (□), N_3 (△) and OH (●) as a function of absorbed dose after γ-irradiation. The solutions were saturated with N_2O and contained $5 \cdot 10^{-5}$ M ferrocytochrome *c* as an initial concentration, 0.1 M NaBr, NaSCN or NaN_3, and 5 mM phosphate buffer (pH 7). The amounts were calculated from ΔA at 520, 550 and 570 nm using published data [17].

from the slopes to be 5.9, 5.7, 6.1 and 0.3, respectively. These values indicate that the efficiency is about 100% for the oxidation by Br_2^-, $(SCN)_2^-$ and N_3, but only about 5% for OH radicals.

The oxidation of cyt-II by these radicals was also observed with pulse radiolysis technique. In Fig. 2 the increase or decrease in the absorbance observed 300 μs after the pulse has been plotted as a function of wavelength for the reaction of cyt-II with Br_2^-. Fig. 2 also shows the difference spectrum calculated from the data of Margoliash and Frohwirt [17] on the assumption that all Br_2^- produced after pulse irradiation ($G = 5.8$) converts cyt-II to cyt-III. Our experi-

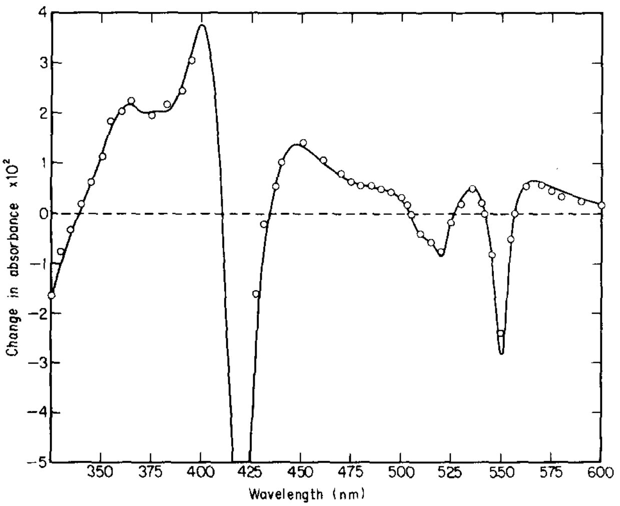
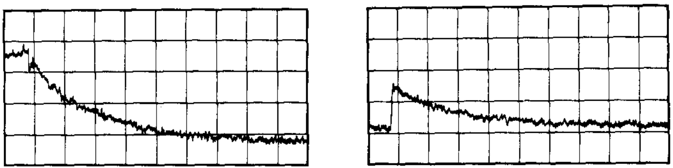
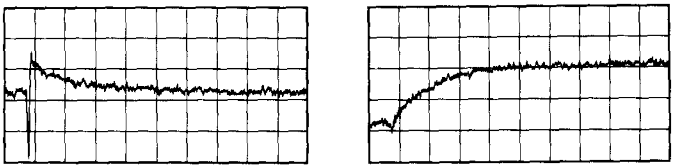


Fig. 2. Change in absorbance 300 μs after pulse due to the reaction of Br_2^- with ferrocytochrome *c*, $1.5 \cdot 10^{-5}$—$3.0 \cdot 10^{-5}$ M; NaBr, 0.05 M; N_2O saturated; pH 7. The open circles are experimental points. The line is calculated from Ref. 17, assuming $G = 5.8$.



(a) Br_2^- at 550nm

(c) $(SCN)_2^-$ at 504nm



(b) Br_2^- at 339 nm

(d) N_3 at 450nm

Fig. 3. Typical examples of transmittance changes as a function of time due to the oxidation of ferrocytochrome *c* by Br_2^-, $(SCN)_2^-$ and N_3 after 0.5 μs pulse. Cyt-II oxidation was monitored at 550 and 450 nm, and radical reduction at 339 and 504 nm. N_2O-saturated solution contained $3.5 \cdot 10^{-5}$—$5.2 \cdot 10^{-5}$ M ferrocytochrome *c*, 0.05 M X^- and 5 mM phosphate buffer (pH 7). (a) Br_2^-, at 550 nm; (b) Br_2^-, at 339 nm; (c) $(SCN)_2^-$, at 504 nm; (d) N_3, at 450 nm. Abcissa scale, 12 μs per division in all cases; ordinate scale, 6.7% transmittance per division for (a), (b) and (c), and 5.7% for (d).

mental points agree with the calculated spectrum. This good agreement in Fig. 2 implies that the conversion of cyt-II to cyt-III occurs with almost the same efficiency of about 100%, as in the case of γ-irradiation. Similar conversion efficiency was obtained for the reaction with $(SCN)_2^-$ or N_3 for the pulse radiolysis. No accurate measurements could be carried out in the range of 395 nm $< \lambda <$ 430 nm under the present conditions because of the high background absorbance of the solution.

Changes in the transmittance as a function of time due to the oxidation of cyt-II by the radicals were found to be mono-phasic. Typical examples are shown in Fig. 3. From the analysis of the kinetic data, it was found that the oxidation of cyt-II by Br_2^-, $(SCN)_2^-$ and N_3 obey the pseudo first-order kinetics, the rates of which were proportional to the cyt-II concentration in the range of $1.5 \cdot 10^{-5}$ M—$6 \cdot 10^{-5}$ M. The linear relationship between apparent first-order rate constants and cyt-II concentration provide second-order rate constants. The rate constants were usually calculated from the transmittance changes at 450 nm and 550 nm for the oxidation by Br_2^- or N_3 and at 550 nm and 565 nm

TABLE I

SECOND-ORDER RATE CONSTANTS FOR THE OXIDATION OF FERROCYTOCHROME *c* BY Br_2^-, $(SCN)_2^-$ AND N_3 RADICALS

Oxidant	k_{obs} ($M^{-1} \cdot s^{-1}$) (X 10^{-8})			k_{calc} ($M^{-1} \cdot s^{-1}$) (X 10^{-8}) *
	pH 7 I = 0.015	pH 7 I = 0.073	pH 8 I = 0.073	pH ~7
Br_2^-	21	9.7	9.0	0.77
$(SCN)_2^-$	15	7.9	8.1	0.18
N_3	14	13	14	1.07

* See the Discussion in the text.

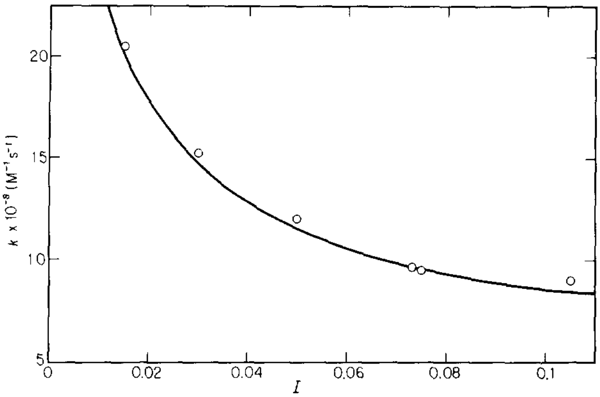


Fig. 4. Dependence of the second-order rate constant on the ionic strength (I) for the oxidation of ferrocytochrome c by Br_2^-. Open circles are experimental points at pH 7. The line is calculated from Eqn. 1. The values of R_1, R_2, Z_1 and Z_2 are given in the text.

for the oxidation by $(SCN)_2^-$ to avoid overlapping of the absorption change of cyt-II and that of the radicals (λ_{max} = 360 nm for Br_2^- [25], 480 nm for $(SCN)_2^-$ [18,26] and 275 nm for N_3 [19,27]). The decays of Br_2^- and $(SCN)_2^-$ due to the reactions with cyt-II were also followed by measurement of their absorptions at 339 nm and 504 nm, respectively (see Fig. 3 (b) and (c)). These wavelengths are isosbestic points for the conversion between cyt-II and cyt-III. The decay rates were identical with those calculated from the absorption changes of cyt-II at 450, 550 or 565 nm. The results obtained at two different pH values and ionic strengths are summarized in Table I. For the oxidation by OH the rate constant was found to be more than $1 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$, but could not be determined accurately since the yield was too small.

In order to determine the effects of the ionic strength on the rates of oxidation by Br_2^-, $(SCN)_2^-$ and N_3, several concentrations of NaBr, NaSCN and NaN_3 were used. The rates of reactions with Br_2^- and $(SCN)_2^-$ decrease with increasing ionic strength and no effect on the rate was observed for the reaction with N_3, as expected from the Brönsted equation. The plots of the second-order rate constants versus ionic strength (I) for the reaction with Br_2^- are shown in Fig. 4. The solid line in Fig. 4 shows the curve calculated from Eqn. 1, derived by Wherland and Gray [28] according to the Marcus theory, in which they took into consideration the coulombic interaction between the reactants and also differences in their sizes. They derived:

$$\ln k = \ln k_\infty - 3.576\left[\frac{\exp - \kappa R_2}{1 + \kappa R_1} + \frac{\exp - \kappa R_1}{1 + \kappa R_2}\right]\left[\frac{Z_1 Z_2}{R_1 + R_2}\right] \qquad (1)$$

where k is the observed rate constant, k_∞ is the rate constant at infinite ionic strength, R_1 and R_2 are the radii of the reactants (16.5 Å for cyt-II and 2.5 Å for Br_2^-), Z_1 and Z_2 are the charges on the reactants (+8 for cyt-II and −1 for Br_2^-), and $\kappa = 0.329\sqrt{I}$ Å^{-1}. When the value of $8 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ for k_∞ is assumed from Fig. 4, the value of $1.6 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$ can be calculated for k_0 using Eqn. 1.

The reaction of cyt-III with Br_2^- was also studied by pulse radiolysis for N_2O-

saturated cyt-III solution containing Br^- at pH 7. The absorption changes observed after the pulse irradiation at 550 nm, 450 nm and several other wavelengths were barely detectable. This result is consistent with that obtained on γ-irradiation for cyt-III solutions. The decay of Br_2^- measured near 360 nm was found to be faster than its disproportionation process. The second-order rate constant for the reaction, $k(\text{cyt-III} + Br_2^-)$, was calculated to be about $5 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ at pH 7, $I = 0.073$ using an approximate method for the kinetic analysis of concurrent first- and second-order reactions [29]. This indicates that Br_2^- can react with cyt-III, perhaps at the sites of the aromatic residues on the protein moiety, although it does not cause conversion of the iron charge. The rate of decay of $(SCN)_2^-$ measured near 500 nm for the reaction with $(SCN)_2^-$ could not be evaluated, since it was comparable with the rate of the bimolecular decay process with itself.

Discussion

The results in the present paper show that under the conditions employed here the reactions of Br_2^-, $(SCN)_2^-$ and N_3 with cyt-II are simple second-order oxidation processes with a yield of about 100% and high rate constants ($k \approx 10^9$ $M^{-1} \cdot s^{-1}$). During the oxidation of cyt-II no detectable intermediate complexes are observed. However, the OH radical, in spite of being a powerful oxidizing agent, oxidizes cyt-II with a very small yield (approx. 5%). This result indicates that most of the OH radicals react with the amino acid residues on the protein surface without leading to oxidation of the ferrous heme.

Several authors have reported that H and OH radicals reduce cyt-III with a yield of about 50% and that an intramolecular process is involved in the reduction process [2,3,30,31]. Thus, radicals formed on the protein surface by the attack of H and OH are capable of reducing the ferriheme buried in the molecule. Van Leeuwen et al. [32] recently demonstrated that the reduction was explicable by an intramolecular electron-tunneling mechanism. For the oxidation of cyt-II by Br_2^-, $(SCN)_2^-$ and N_3 we examined a similar possible mechanism by which intramolecular electron transfer takes place from the heme to an electron-deficient site on the protein surface produced by the attack of the radicals. The observed values of the rate constants for the oxidation were compared with the rate constants calculated on the basis of the same assumption as made by Masuda et al. [33] for the reaction with OH. They reported that the observed rate constant for the reaction of cyt-III with OH is consistent with a value obtained by the summation of the rate constants of OH towards the free amino acids, corrected, however, according to their degree of exposure to the surface. The calculated rate constants (k_{calc}) in Table I were evaluated by summation of the product of a_i and k_i; where a_i signifies the sum of the steric correction factors for the amino acid residue of type i on the protein as estimated by Masuda et al. [33] for the reaction with OH, and k_i corresponds to the rate constant of the X_2^- or N_3 radical reaction with the free amino acids. The latter values were determined by Adams et al. [9] for Br_2^- and $(SCN)_2^-$, and by Land and Prütz [16] for N_3. The value of the rate constant, $5 \cdot 10^7$ $M^{-1} \cdot s^{-1}$, for the reaction of cyt-III with Br_2^- obtained here supports the estimation for the k_{calc} ($7.7 \cdot 10^7$ $M^{-1} \cdot s^{-1}$), since Br_2^- is expected to react only at sites on the protein moiety of cyt-III. It should be noted here that smaller values of the steric correction factors should be estimated for the reactions with Br_2^-, $(SCN)_2^-$ and N_3 than that for the reaction with OH, since the sizes of Br_2^-, $(SCN)_2^-$ and N_3 are larger than that of OH. This implies that each value of k_{calc} gives an upper limit. The observed rate constant, k_{obs}, at neutral pH is, nevertheless, evidently more than 10-times larger than k_{calc} (shown in Table I) for each reaction with Br_2^-, $(SCN)_2^-$ or N_3.

It has been published that the rates of reactions of Br_2^- and $(SCN)_2^-$ with cysteine [9] and methionine and of the reaction of N_3 with cysteine and tyrosine [16] markedly increase under alkaline conditions.

The rates of reaction of the radicals with the residues of cysteine, tyrosine and methionine must contribute to a great extent to k_{calc} under the present assumption. The redox potential of cyt-II/cyt-III does not change appreciably in the pH range 6.7—9.0 [34]. Hence, if the radicals simply react only with amino acid residues on the protein surface, an increase in the rate of the oxidation is expected in alkaline solutions, although the p*K* values of the amino acid residues in the protein might be shifted from those of the free amino acid. As shown in Table I, no substantial difference was observed at the identical ionic strength between the rate constants at pH 7 and 8.9 for three radicals.

It is reasonable to propose, therefore, that Br_2^-, $(SCN)_2^-$ and N_3 radicals react at a more reactive and specific site, in which the partially exposed edge of the porphyrin ring rather than amino acid residues on the surface of the protein is probably involved. It was suggested that the redox reactions of cytochrome *c* with many nonphysiological metal complexes take place at a common site, close to the exposed heme edge [1,28,35—37]. The reduction of cyt-III by CO_2^- [6] and by various pyridinyl radicals [8] was also proposed to take place at such a site and proceed with high rate constants ($k = 2 \cdot 10^8 — 1 \cdot 10^9\ M^{-1} \cdot s^{-1}$). Those rate constants are very similar to that of the reaction with hemin (III)-C [38], in spite of the fact that the reaction site in cyt-III corresponds to only several percent of the total surface. These reduction reactions are similar to the reactions with Br_2^-, $(SCN)_2^-$ and N_3 radicals with respect to the high reactivity and the reaction site, although the redox directions are reversed.

There are many studies reported on the reactions of heme proteins with small-size redox agents, in which the charges derived from the ionic-strength dependence of the rate constants according to the Brönsted-Debye fomula are concluded to reflect the localized charges at the reaction sites on the proteins [6,39,40]. However, a comparative study of the reduction of two differently charged cytochromes *c* carried out by Feinberg et al. [41] demonstrated that the charge derived using Eqn. 1 reflect the overall surface charge of the heme protein. As shown in Fig. 4, the ionic-strength dependence of the observed rates fits fairly well the theoretical curve which originates from the Marcus theory, when the charge of cyt-II is taken to be +8, corresponding to the overall surface charge of cyt-II in a neutral solution [42]. The result also supports our proposal that the oxidation of cyt-II by Br_2^- radicals proceed by remote attack, probably on the partially exposed region of the heme.

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