Application of Promoter Modified Electrodes to Bioelectrochemical Measurements on the Effects of Origin and Modification of Lysine Residues of Cytochrome *c*

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Using promoter-modified electrodes, electron-transfer reactions of cytochrome *c* at an electrode and with cytochrome *c* oxidase were examined. The electrochemical behavior of cytochromes *c* from horse, bovine, chicken and tuna hearts at an electrode was similar to each other. Little difference was observed for the reactions between cytochromes *c* of various origins and cytochrome *c* oxidase from bovine in a phosphate buffer solution. Also, when a few (less than three) lysine residues of cytochrome *c* were treated with 4-chloro-3,5-dinitrobenzoic acid (CDNP) or 2,4-dinitrofluorobenzene (DNP), the electrode reaction of the modified (especially, mono-substituted) cytochrome *c* still showed no significant difference from that of the native one. On the other hand, the electron-transfer reaction between CDNP- or DNP-substituted cytochrome *c* at lysine 13 and/or 72 and cytochrome *c* oxidase was greatly affected, even when only one or two lysine residues of cytochrome *c* were modified. Since the cytochrome *c* molecules of different origins examined have about 3 – 19 differences in their amino acid sequences, but most lysine residues remain invariant, the present results indicate that lysine residues of cytochrome *c* play an important role in the interaction with cytochrome *c* oxidase; however, the promoter modified electrode surface does not recognize cytochrome *c* molecule very rigorously. The present study also shows that electrochemical techniques with the aid of functional electrodes are applicable as useful, convenient methods to analyze the biological reactions of proteins.

Keywords Cytochrome *c*, cytochrome *c* oxidase, promoter modified electrode, electron transfer reaction, respiratory chain, molecular recognition

Protein electrochemistry has been extensively developed from pure and applied scientific points of view in recent years.[1-11] For such proteins as cytochrome *c*[1-6], ferredoxin[5,6,10], plastocyanin[6] and azurin[6], the functional electrodes on which rapid direct electron-transfer reactions of proteins take place have been developed. Promoter-modified[4] and indium oxide[3,5] electrodes are typical instances for cytochrome *c*, which is a low molecular weight (*ca.* 12000) heme protein having a function of electron transport in the respiratory chain. These functional electrodes make it possible to examine bioelectrochemical measurements of proteins by the usual electrochemical and spectroelectrochemical techniques: for example, a pH-temperature diagram for state changes of ferricytochrome *c* was prepared[12] on the basis of changes in the formal redox potential with the pH and temperature. *In situ* circular dichroism (CD) spectra of cytochrome *c* have also been obtained[13,14] to estimate the conformational changes of cytochrome *c* associated with electron-transfer reactions.

Also, the reactions of proteins in solution with their physiological and enzymatic redox partners can be examined electrochemically by monitoring the electrocatalytic currents using suitable systems. In fact, the application of ferredoxin electrochemistry to bioelectrochemical carbon dioxide fixation on the basis of enzyme reactions was demonstrated.[10] Similarly, the reaction between cytochrome *c* and its oxidase was analyzed by using an electrochemical model system of the terminal of the respiratory chain.[15,16]

Since amino acid residues of the protein surface, in general, have been considered to play an important role in the physiological electron transfer reactions, in the present study the effects of the origin and modification of amino acid residues of cytochrome *c* on both the electrode reaction and the electron-transfer reaction with its oxidase were examined electrochemically in order to understand more precisely the role of the charge of the protein surface on these electron-transfer reactions.

Experimental

Cyclic voltammetry of cytochrome *c* was carried out at 25°C in a phosphate buffer (PB) solution with 0.1 M NaCl (pH 7) using a Toho Giken 2020/2230 potentiostat with a function generator. Promoter-modified gold (wire of 5 mm length and 0.5 mm diameter) electrodes, the surfaces of which had been polished with a 0.05 μm alumina slurry, and then modified with bis(4-pyridyl)-

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disulfide (PySSPy) or 6-mercaptopurine (PuSH), were used as working electrodes;[4,13] no significant difference in the electrochemical behavior between these modified (PySSPy-Au and PuSH-Au) electrodes was observed in the present experiments. The counter electrode was a platinum plate; an Ag/AgCl (saturated KCl) was used as a reference electrode to which all potentials given in this paper are referred.

Cytochromes *c* from horse (type VI), bovine (type V), chicken (type X) and tuna (type XI) hearts were purchased from Sigma, and purified by column chromatography on a Whatman CM-32 column at 4° C as described previously[17], according to the literature.[18] The purity of cytochrome *c* was estimated from the ratio of absorbance ($A_{ox}^{280}/A_{red}^{550}$) at 280 and 550 nm for oxidized and reduced forms, respectively, to be 1.25 (1.05 for tuna cytochrome *c*).[18] The concentrations of purified cytochromes *c* of all origins used were determined for the reduced form using the molar absorption coefficient at 550 nm (29500 M^{-1} cm^{-1}).[19] Cytochrome *c* oxidase (Sigma, from bovine heart) was used as received. The difference in the molar absorption coefficient, ($\Delta\varepsilon$=164 mM^{-1} cm^{-1} at 445 nm[20]), between the reduced and oxidized forms was used to evaluate the concentration. All other reagents were of analytical grade and sample solutions were prepared with Millipore Milli-Q grade (ρ>17 MΩ cm) water.

When lysine residues of cytochrome *c* were modified, purified horse heart cytochrome *c* was treated at room temperature with 4-chloro-3,5-dinitrobenzoic acid (CDNP) or 2,4-dinitrofluorobenzene (DNP), both of which are specific reagents for the selective modification of lysine residues of cytochrome *c*, by procedures similar to those given in the literature.[21-25] For the modification of CDNP or DNP, 400 mg (*ca.* 30 μmol) of purified cytochrome *c* was reacted for 6 h in a 0.2 M borate buffer solution (pH 9, 3 ml)[22,23] containing *ca.* 33 μmol of CDNP, or in a 0.1 M phosphate buffer (pH 7.8, 4 ml)[24,25] with *ca.* 80 μmol of DNP, respectively. After CDNP (or DNP) remained unreacted it was removed by gel filtration using a phosphate buffer solution (pH 7.8) as an eluent on a Sephadex G-25 column (1.5×45 cm); the mixture of various CDNP (or DNP) modified and native cytochrome *c* molecules was separated into several fractions by cation-exchange chromatography on a Whatman CM-32 column (1.9×100 cm) using a phosphate buffer solution (pH 7.8) with the aid of a gradient elution technique at a flow rate of *ca.* 8.5 ml/h, giving CDNP (or DNP) modified cytochromes *c*. The elution profile was obtained by spectroscopically: during the separation procedure, every 10 ml of the eluted solution was collected, and a part of each solution was reduced with sodium dithionite in order to monitor the absorbance at 550 nm (Fig. 1). The obtained elution profiles were similar to those previously reported.[21-23] Several fractions in the same peak on the chromatogram, which have similar properties to each other, were put together, when necessary, to obtain larger sample amounts.

The numbers (N) of CDNP (or DNP) immobilized per molecule of cytochrome *c* were estimated spectroscopically[21,22] using the specific absorbance peaks of the modifiers from the difference in the absorbance at 450 nm (for CDNP) or 360 nm (for DNP) between the modified cytochrome *c* and native one by the following equation:

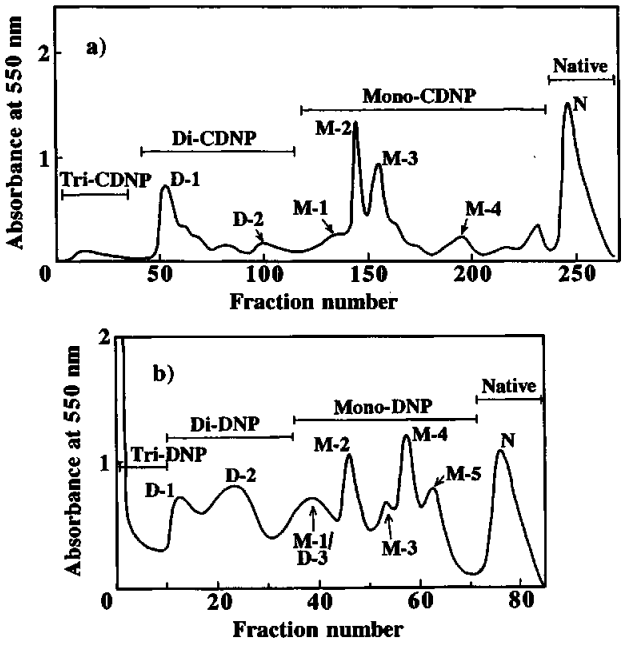

Fig. 1 Typical elution profiles of a) CDNP- and b) DNP-modified cytochrome *c* during the separation on a Whatman CM-32 column (see the text for details). Several fractions with labels were used in the present experiments.

$$N = \text{CDNP(or DNP)/cytochrome } c = \frac{[A_{ox}^{\lambda_m} - (\varepsilon_{ox}^{\lambda_m}/\varepsilon_{red}^{550})A_{red}^{550}]/\varepsilon_m^{\lambda_m}}{A_{red}^{550}/\varepsilon_{red}^{550}}, \quad (1)$$

where $A_{ox}^{\lambda_m}$ and A_{red}^{550} are the absorbances of the oxidized and reduced forms of modified cytochrome *c*, respectively, at the wavelengths shown as superscripts. The modifiers (CDNP and DNP), themselves, have no influence on the spectra of modified cytochromes *c* at 550 nm. λ_m is the wavelength taken for the modifiers (450 nm for CDNP and 360 nm for DNP) and $\varepsilon_m^{\lambda_m}$ is the molar absorption coefficient of the modifier at λ_m (5.0×10^3 for CDNP and 1.8×10^4 M^{-1} cm^{-1} for DNP). $\varepsilon_{ox}^{\lambda_m}$ and ε_{red}^{550} (=29500 M^{-1} cm^{-1})[19] indicate the molar absorption coefficients at the wavelengths shown as superscripts of the oxidized and reduced forms of native cytochrome *c*. The values of $\varepsilon_{ox}^{\lambda_m}/\varepsilon_{red}^{550}$ are 0.463 at λ_m=450 nm and 1.00 at λ_m=360 nm (see Fig. 2). Thus, the N value can be evaluated by measuring the spectra of the oxidized, and then reduced (by adding sodium dithionite), forms of the modified cytochrome *c*. The numbers of the immobilized CDNP (or DNP) used were less than three, because any further modification of the amino acid residues of cytochrome *c* would change their structures more seri-

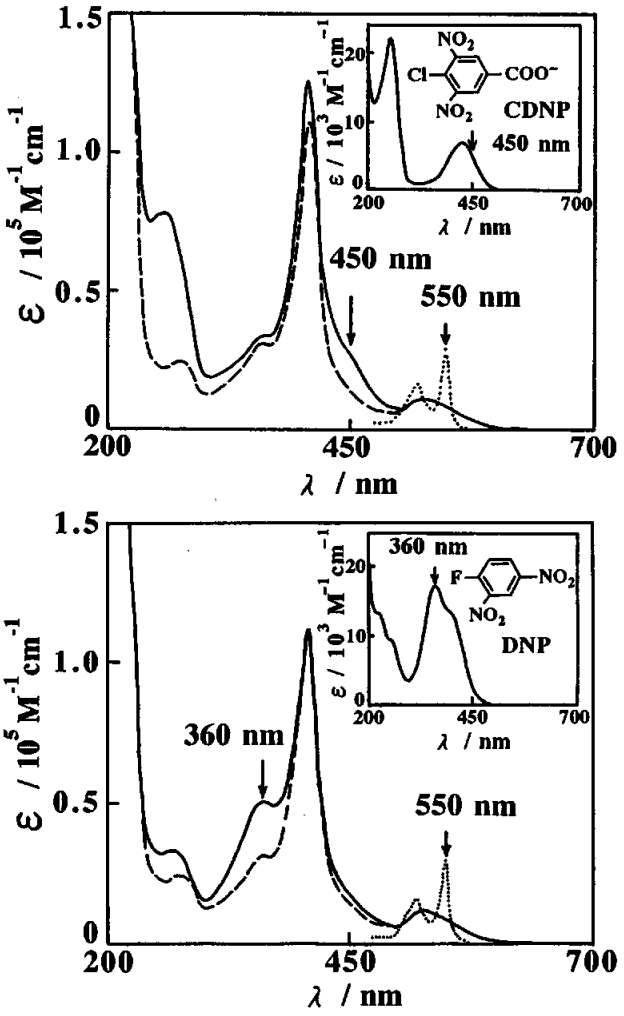

Fig. 2 Typical spectra of a) CDNP- and b) DNP-modified ferricytochrome *c* (——), together with native one (----) and after reduction (····) of the modified cytochrome *c* with sodium dithionite. The spectra of modifiers are also given as insets.

ously.

In the present study, no further precise separation was made to distinguish each site specific component of the modified cytochrome *c* completely. However, according to the literature[21,22], among the CDNP monosubstituted cytochrome *c*, the fastest coming out fraction (M-1 in Fig. 1a) likely contains CDNP-modified cytochrome *c* at lysine 13 (K13/CDNP-Cyt. *c*) as a main component. The following fraction (M-2) would correspond to that modified at lysine 72 or a mixture of it with K13/CDNP-Cyt. *c*. Fraction M-3 is a mixture[22] of CDNP singly modified cytochrome *c* molecules at such lysine residues as 86, 87, 8, 27 and 73. Fraction M-4 has been reported[22] to contain the species of which lysines at the back side of the heme edge, such as lysine 60, are modified. Some of the fractions shown in Fig. 1a, however, gave too small amounts of samples in the present work to obtain reliable results. Fractions D-1 and D-2 in Fig. 1a are mixtures of CDNP doubly modified cytochrome *c* at two positions of such lysine residues as 13, 86, 87, 8, 27, 73 and 60. Taking into account the order of reactivity of CDNP with lysine residues (72>13>60>others)[22] of cytochrome *c* and the elution profile[22], CDNP doubly modified cytochrome *c* at lysines 13 and 72 for fraction D-1 and those at 13 (or 72) and 60 for fraction D-2 would be the main components. On the other hand, for DNP-modified cytochrome *c*, it is difficult to distinguish the substituted sites, at present, because of a lack of data. However, since lysine 13 has been reported[24] to be the most reactive site for such a reagent, the first fraction among the DNP mono-substituted cytochrome *c* would again be the lysine 13 modified one. Also, similarities, to some extent, of the reaction sites of cytochrome *c* and of elution profiles between DNP and CDNP may be assumed.

Results and Discussion

Effect of origin of cytochrome c

The amino acid sequences of cytochromes *c* from various origins are well established[26], as shown in Table 1, and there are 3 - 19 differences, depending on their origins, between the cytochrome *c* molecules examined. Figure 3 shows typical cyclic voltammograms of cytochromes *c* of different origins in a phosphate buffer solution with 0.1 M NaCl (pH 7) at 25°C. No significant influence of the origin of cytochrome *c* on the electrochemical behavior at promoter modified electrodes (PySSPy-Au and PuSH-Au) was observed: the formal redox potentials ($E^{\circ\prime}$) obtained as a midpoint of anodic and cathodic peak potentials were 68(±3) mV for horse, bovine and chicken cytochrome *c* and *ca.* 63(±3) mV *vs.* Ag/AgCl for tuna cytochrome *c*. Both the heterogeneous electron-transfer rate constant (k_{sh}=*ca.* 5×10^{-3} cm/s)[27,28] and the diffusion coefficient (D=*ca.* 1×10^{-6} cm^2/s)[28] of cytochromes *c* of different origins, which were estimated, respectively, from the scan-rate dependence of the peak separation[27,28] and from the cathodic peak currents of cyclic voltammograms at various scan rates[28], are also similar to each other. Using an In_2O_3 electrode[5], almost the same results as those observed at promoter modified electrodes were again obtained. Also, the CD spectra of cytochromes *c* of different origins were very similar to each other, indicating that the structures of these cytochromes *c* are essentially the same. Thus, the difference in the amino acid sequence of cytochrome *c* examined does not greatly affect the electrochemical behavior under the present experimental conditions.

Using a promoter-modified electrode, the electron-transfer reaction between cytochrome *c* and its oxidase can also be estimated by monitoring the catalytic current using an electrochemical model system[16] of the terminal of the respiratory chain: *i.e.*, promoter modified electrode/cytochrome *c*/cytochrome *c* oxidase/dioxygen. For physiological electron transfer reactions of proteins, the charge of the amino acid residues of the protein

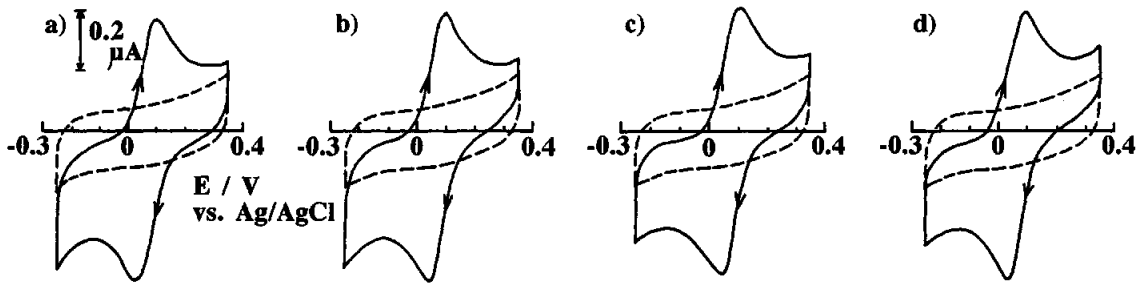

Fig. 3 Cyclic voltammograms of *ca.* 100 μM cytochromes *c* of different origins (a: horse; b: bovine; c: chicken; d: tuna) at a PySSPy-Au electrode in a phosphate buffer solution with 0.1 M NaCl (pH 7) at 25°C. Scan rate: 20 mV/s. ----: background.

Table 1 Amino acid sequences of cytochrome *c* molecules of different origins[26] using single letter amino acid codes while indicating the differences of the amino acid residues as boxes

		10	20	30	40	50	60
Horse	(1)	GDVEKGKKIFVQKCAQCHTVEKGGKHKTGPNLHGLFGRKTGQAPGFTYTDANKNKGITWK					
Bovine	(2)	GDVEKGKKIFVQKCAQCHTVEKGGKHKTGPNLHGLFGRKTGQAPGFSYTDANKNKGITWG					
Chicken	(3)	GDIEKGKKIFVQKCSQCHTVEKGGKHKTGPNLHGLFGRKTGQAEGFSYTDANKNKGITWG					
Tuna	(4)	GDVAKGKKIFVQKCAQCHTVENGGKHKVGPNLWGLFGRKTGQAEGYSYTDANKSKGIVWN					

		70	80	90	100
	(1)	EETLMEYLENPKKYIPGTKMIFAGIKKKTEREDLIAYLKKATNE			
	(2)	EETLMEYLENPKKYIPGTKMIFAGIKKKGEREDLIAYLKKATNE			
	(3)	EDTLMEYLENPKKYIPGTKMIFAGIKKKSERVDLIAYLKDATSK			
	(4)	NDTLMEYLENPKKYIPGTKMIFAGIKKKGERQDLVAYLKSATS-			

surface plays an important role. Thus, the reaction between cytochrome *c* and its oxidase may change when proteins of different origins are used. By using cytochromes *c* oxidase from bovine, reactions between cytochromes *c* from different origins and the oxidase were compared (Fig. 4). The catalytic current observed, however, showed no significant difference in a phosphate buffer solution at an ionic strength of 0.1 M (Fig. 4b). Only when in a Good's HEPES buffer at a very low ionic strength was used (see Fig. 4c), did the catalytic current change in the order bovine>horse≫chicken and tuna cytochrome *c* for the reaction with bovine cytochrome *c* oxidase. These results can be explained as follows. Although the amino acid sequences of cytochromes *c* of different origins (horse, bovine, chicken and tuna) have 3 – 19 differences to each other, lysine residues (shown as K in Table 1) are almost invariant for all of the cytochrome *c* molecules used; only limited lysine residues, like lysine 60, which are not near to the heme edge, but to the back side of the molecule, are different. Thus, the electron-transfer reactions of cytochrome *c* at an electrode and with its oxidase appeared to be almost independent of the origin of cytochrome *c*. On the other hand, in a solution of low ionic strength, an electrostatic interaction between cytochrome *c* and its oxidase becomes predominant; also, the HEPES is a so-called non-binding type buffer solution, meaning that no contained ion binds specifically to proteins. Under such conditions, although the interaction between cytochrome *c* and the oxidase became more sensitive to give the differences (Fig. 4c), the electrode reaction of cytochrome *c* at a promoter-modified electrode did not change very much (not shown).

Effect of a modification of lysine residues

The CDNP- and DNP-modified horse heart cytochrome *c* molecules prepared in the present study showed clear voltammetric responses (Fig. 5). The CDNP mono-substituted cytochrome *c* molecules showed a slight change in the $E^{\circ\prime}$ and k_{sh} values. As the number of modified CDNP increased to be two or more, the apparent $E^{\circ\prime}$ value of the modified cytochrome *c* shifted toward negative potentials and the k_{sh} value decreased more (Fig. 6a). Similar results were again observed when cytochrome *c* was modified with DNP (Fig. 6b), where the introduction of DNP caused a slight larger

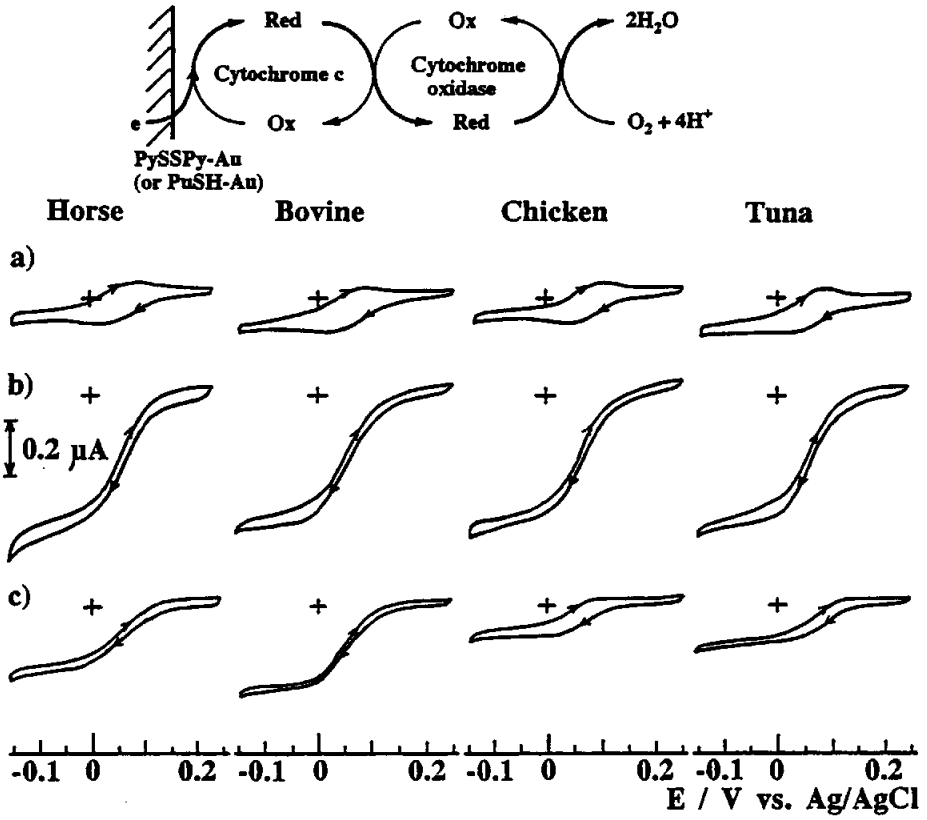

Fig. 4 Typical cyclic voltammograms of cytochromes *c* of different origins (horse; bovine; chicken; tuna) at a PySSPy-Au electrode at 25°C. a) 100 μM cytochrome *c* in a phosphate buffer solution (ionic strength of *ca.* 0.1 M, pH 7) under nitrogen; b) 86 μM cytochrome *c* in the presence of 0.1 μM cytochrome *c* oxidase (from bovine) and dioxygen in the same buffer solution as a); c) same as b) except in a 1/1000 M HEPES buffer solution (pH 7). Scan rate: 1 mV/s. A scheme for the electrochemical model of the terminal of the respiratory chain is also given at the top.

negative shift in the $E^{\circ\prime}$ value than did CDNP-modified cytochrome *c*. CDNP introduces a negative charge due to the dissociated carboxylic group at the positively charged amino group of the lysine residue, while DNP changes the positive charge to be neutral. Thus, the similarity in the electrochemical behavior between CDNP and DNP modified cytochromes *c* suggests that the charge of cytochrome *c* does not play a very important role in the electron-transfer reaction at a promoter-modified electrode under the present experimental conditions, although by a modification of the lysine residues, the $E^{\circ\prime}$ values shifted to be more negative and the k_{sh} values were reduced to be about one-half of the initial value. The CD spectra of the modified cytochrome *c* molecules showed smaller negative peaks around 220 nm than that of native cytochrome *c*, indicating that the entire protein structure of modified cytochrome *c* became somewhat disordered; the negative peak of DNP-modified cytochrome *c* was smaller than that of the CDNP-modified one (not shown), suggesting that the DNP modification caused a larger structural change than did the CDNP modification. This is probably because DNP is more hydrophobic than CDNP, and that DNP may enter into the protein molecule. Since ferrocytochrome *c* has a more compact and stable conformation than does ferricytochrome *c*[12,29] (this was also confirmed to be valid by CD measurements for the modified cytochromes *c*), the slightly negative shift in the $E^{\circ\prime}$ value may be attributable to the possibility that the heme moiety of modified ferricytochrome *c* was a slightly more exposed to the solvent so as to be more stabilized than native cytochrome *c*.

In order to examine the role of lysine residues of cytochrome *c* on the electron-transfer reaction with cytochrome *c* oxidase, the catalytic currents were also measured for the electrochemical model of the respiratory chain using modified cytochrome *c* molecules. The cathodic current of the modified cytochrome *c*, itself, slightly decreased with an increase in the number of introduced CDNP or DNP (Fig. 7), as was reported by other workers[23] for CDNP-modified cytochrome *c*: this may be partly because the modified cytochrome *c* contained a few associated molecules, such as a dimer having a smaller diffusion coefficient. More interestingly, the electron-transfer reaction between the modified cytochrome *c* and cytochrome *c* oxidase in the solution was

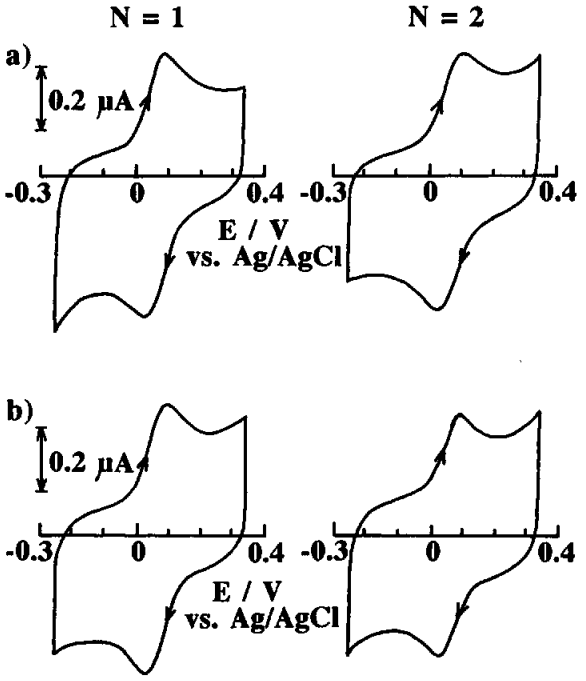

Fig. 5 Examples of cyclic voltammograms of a) CDNP- and b) DNP-modified horse heart cytochrome *c* at a PySSPy-Au electrode in a phosphate buffer solution with 0.1 M NaCl (pH 7) at 25°C. The results obtained using fractions of M-2 and D-2 in Fig. 1 for both CDNP- and DNP-modified cytochrome *c* are given for N=1 and N=2, respectively. Scan rate: 20 mV/s.

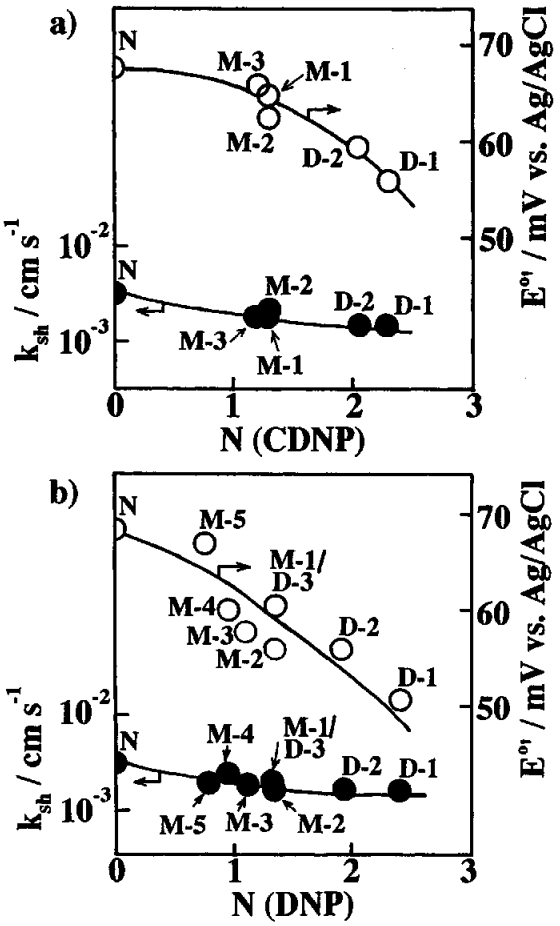

Fig. 6 Changes in the k_{sh} (in logarithmic scale) and $E^{o\prime}$ values of a) CDNP- and b) DNP-modified horse heart cytochrome *c* as a function of the number (N) of immobilized modifiers. From which fractions in Fig. 1 the data were obtained are noted by the symbols. The deviation from integral values for the N value would be partly because the samples used were not pure mono- and di-substituted molecules.

remarkably inhibited, even when mono-substituted modified cytochrome *c* was used, as shown in Fig. 7, where the ratios of the catalytic currents to non-catalytic peak currents of the modified cytochrome *c*, i_c/i_{pc}, are given. It is clear that i_c decreased more significantly than did i_{pc} itself: a modification of lysine 13 and/or 72 with CDNP seems to have a greater affect than other lysine residues (compare, for example, points M-1 and/or M-2 with M-3 in Fig. 7a). Although the data were somewhat scattered, the M-4 fraction of CDNP mono-substituted cytochrome *c* showed much larger i_c/i_{pc} values (not shown) than did other fractions, such as M-1, M-2 and M-3, indicating that the modification of lysine 60 had a smaller affect on the reaction with cytochrome oxidase. These results are in good agreement with that reported[21,22] from biological measurements on kinetics. A similar decrease in the i_c/i_{pc} ratio was also obtained when DNP-modified cytochrome *c* was used, but, clearly, the inhibition was less than in the case of the CDNP-modified cytochrome *c* (see Figs. 7a and b). Since the positions of the lysine residues modified with CDNP may not correspond completely to those modified with DNP, it is not very easy to discuss the difference between the CDNP- and DNP-modified cytochrome *c* in detail. However, when we compare points M-1 and/or M-2 in Fig. 7 for CDNP- and DNP-modified cytochrome *c* molecules, where the lysine 13 or 72 residue was very likely modified, the reactivity with cytochrome *c* oxidase was clearly smaller for CDNP-modified cytochrome *c* than for the DNP-modified one. This would be attributable to a difference in the charge of the modifiers; an electrostatic interaction is suggested to be important for the protein-protein reaction.

By using the simplified pseudo-first-order reaction scheme, $O+e \rightleftharpoons R$, $R+Z \longrightarrow O$, where O and R are ferri- and ferrocytochrome *c*, Z is the oxidized form of cytochrome *c* oxidase; its concentration ([Z]) is assumed to remain constant in the presence of sufficient amounts of dioxygen and, thus, the first-order rate constant (k_f) is given by $k_f=k[Z]$. The observed catalytic currents were analyzed in order to obtain approximate kinetic information from the i_c/i_{pc} ratio using the Nicholson's procedure.[16,30] Nevertheless, the present experimental conditions and the included enzyme reaction are not fully satisfactory for the simplified first-order reaction scheme; the k_f values obtained at an ionic strength of *ca.* 0.1 M were, as a criterion, roughly constant, and independent of scan rates examined (<20 mV/s), thus

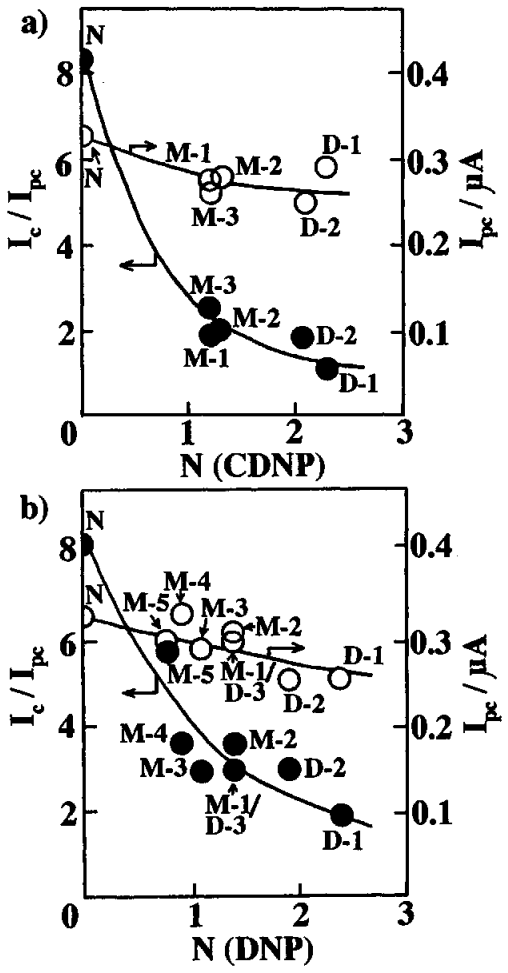

Fig. 7 Changes in the i_{pc} and i_c/i_{pc} values as a function of the number (N) of immobilized modifiers for a) CDNP- and b) DNP- modified horse heart cytochrome c in a phosphate buffer solution with 0.1 M NaCl (pH 7) at 25°C. The peak currents (i_{pc}) were obtained for *ca.* 100 μM cytochrome c at a scan rate of 20 mV/s under nitrogen, while the catalytic currents (i_c) were obtained for 86 μM cytochrome c in the presence of both 0.1 μM cytochrome oxidase (from bovine) and dioxygen at a scan rate of 1 mV/s. The data shown are the average values of at least two independent measurements using PySSPy-Au and/or PuSH-Au electrodes.

indicating that the simplified pseudo-first-order reaction scheme may be applicable as reported[31] for similar reactions. From the observed catalytic currents the apparent second-order rate constant (k) of the homogeneous reaction between native cytochrome c and the oxidase was estimated as being $k_f/[Z]$, where [Z] is the concentration of the cytochrome c oxidase used (*ca.* $4.0(\pm 1.0)\times 10^6$ M^{-1} s^{-1}), which is about two orders of magnitude larger than that of physiological reaction at intact mitochondria ($<10^4$ M^{-1} s^{-1})[32], since the former reaction takes place in solution, while the latter takes place at a cell membrane.

In conclusion, the present results indicate that the molecular recognition of cytochrome c is much more rigorous for the protein-protein interaction with cytochrome c oxidase than for the electron-transfer reaction at an electrode. Also, using functional electrodes, electrochemical techniques become powerful and convenient means to analyze the physiological electron-transfer reactions.

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