Polarographic Catalytic Currents Produced by Cytochromes c in Ammoniacal Buffers Containing Cobalt Ions. I. Significance of Heme Group

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Polarographic behavior of the Brdička current (catalytic current produced by proteins in ammoniacal buffers containing cobalt ions) of cytochromes c from five sources (horse, chicken, and tuna hearts, *C. krusei* and *S. cerevisiae*) and of modified horse cytochromes (metal-substituted, metal-free, and heme-free cytochrome, and carboxymethylated cytochrome at methionyl residues) was investigated at dme and hmde. The results indicate that the heme group and its sixth ligand are important in determining the Brdička current-activity of cytochromes c. Replacement of the sixth heme ligand from Met to Lys reduced the Brdička current-activity by about one-half. Brdička current of *D. vulgaris* cytochrome c_3 was interpreted as due to four Lys-liganded heme groups.

Polarographic catalytic currents produced by cytochrome c in ammoniacal buffers containing cobalt ions have been studied by Carruthers[1] and Carruthers and Suntzeff[2] with a dropping mercury electrode (dme) and by Kuznetsov[3] with a hanging mercury drop electrode (hmde). They attributed the catalytic activity of cytochrome c to the cysteine residues in its molecule, as usually recognized with SS- and/or SH-containing proteins. In cytochrome c, however, the two residues of cysteine are linked to the heme group to form heme c and are not present as SS- or SH-groups. We have investigated the polarographic and voltammetric behavior of the catalytic currents, *i.e.* the Brdička currents, of cytochromes c from five sources (horse, chicken, and tuna hearts, *Candida krusei*, and *Saccharomyces cerevisiae*) and of modified horse heart cytochromes (metal-substituted, metal-free, and heme-free cytochrome, and carboxymethylated cytochrome at methionyl residues) and found that the heme group and its sixth ligand are important in the Brdička current-activity of cytochrome c. Brdička current-activity of *Desulfovibrio vulgaris* cytochrome c_3 was interpreted as due to four Lys-liganded heme groups.

Experimental

Materials. Horse heart cytochrome c (horse cyt-c), chicken heart cytochrome c (chicken cyt-c), tuna heart cytochrome c (tuna cyt-c), *Candida krusei* yeast cytochrome c (*C. krusei* cyt-c), and *Saccharomyces cerevisiae* yeast cytochrome c (*S. cerevisiae* cyt-c) were products of Sigma Chemicals Co. The purity of these cytochromes c was checked by means of cellulose acetate membrane electrophoresis. Stock solutions, usually 0.5% in cytochrome c were stored at 5 °C. The concentrations were checked spectrophotometrically.[4] Carboxymethylated cytochrome c (CM-cyt-c) was prepared by carboxymethylation of methionyl residues of horse cyt-c by the method of Babul and Stellwagen, identified by absorption spectroscopy, and its concentration determined spectrophotometrically with an absorbance coefficient 127 $mM^{-1}\,cm^{-1}$ at 406 nm.[5] Porphyrin cytochrome c (porphyrin cyt-c) was prepared by the method of Robinson and Kamen.[6] The product was further purified by the gel filtration method according to Dickerson and Chien.[7] The purified porphyrin cyt-c was identified by absorption spectroscopy, and its concentration determined spectrophotometrically with an absorbance coefficient 81 $mM^{-1}\,cm^{-1}$ at 404 nm.[8] Since porphyrin cyt-c is unstable,[7] freshly prepared porphyrin cyt-c was used for electrochemical measurements within the day. Cobalt cytochrome c (Co-cyt-c) was prepared by the method of Dickerson and Chien, identified by absorption spectroscopy, and its concentration determined spectrophotometrically with an absorbance coefficient 106 $mM^{-1}\,cm^{-1}$ at 426 nm.[7] Apocytochrome c (apo-cyt-c) was prepared by the method of Stellwagen and Rysavy.[9] The apoprotein exhibited no absorption in the visible region, indicating the absence of heme moiety. The concentration of apo-cyt-c was determined spectrophotometrically with an absorbance 0.92 ml $mg^{-1}\,cm^{-1}$ at 277 nm.[9] The purity of all these modified cytochromes was further checked by means of SDS-disk electrophoresis. *Desulfovibrio vulgaris* Miyasaki cytochrome c_3 (*D. vulgaris* cyt-c_3) supplied by T. Yagi was used. All other chemicals used were of reagent grade.

Instruments. Voltage sweep voltammograms and cyclic voltammograms at hmde were recorded on a Yanagimoto P8-MS6C polarograph and a Riken Denshi F42C X-Y recorder or a Yanagimoto PE21-TB2S potentiostat with a built-in sweep voltage generator and a Yokogawa 3077 X-Y recorder. A Metrohm E410 mercury drop electrode was used, its surface area being 0.0187 ± 0.0003 cm^2. D.c. polarograms were recorded on a Yanagimoto P8 polarograph. In most cases a dme having the characteristics $m=1.43$ mg/s and $\tau=4.6$ s at open circuit in ammoniacal buffer at 74 cm height of mercury reservoir (h) was used. In some cases a dme having the characteristics $m=1.60$ mg/s and $\tau=6.3$ s at $h=70$ cm was used.

Electrochemical Measurements. All measurements were made in an H-type cell. All potentials were measured against a saturated calomel electrode (SCE). A buffer solution containing 0.1 M NH_3, 0.1 M NH_4Cl, and 0.1 M KCl (pH 9.4, ionic strength 0.2 mol/dm^3) was used as the base solution. 10 ml of the base solution was transferred to the cell and deaerated by passing nitrogen gas through the solution, which had been previously passed through a solution of the same composition as the base solution. An aliquot of each of the stock solutions of 0.05 M $Co(NH_3)_6Cl_3$ and 0.5% cytochromes c was then introduced into the base solution with a microsyringe under nitrogen atmosphere. In the measurements with hmde a mercury drop freshly formed at the tip of hmde was exposed to the test solution for a given period of time, referred to as exposure time (t_{exp}), during which the electrode potential was kept controlled at the initial voltage (E_i), voltammo-

grams being then run from $E_i = -0.1$ V with a sweep rate, $v = -0.2$ V/s. In determining the current intensities of the catalytic currents an extrapolated theoretical trace of degradating curve of the cobalt peak was subtracted from the current measured at the proper potential. In the measurements with dme, current intensities at the maximum growth of each mercury drop were taken and the catalytic currents measured from the cobalt limiting current. The measurements were carried out at 25 °C unless otherwise stated.

Results

Brdička Current of Horse Cyt-c. Horse cyt-c gave characteristic catalytic current, *viz.*, Brdička current in ammoniacal buffers containing hexaamminecobalt-(III) ion, Co(III), at both dme and hmde. Figure 1 shows d.c. polarograms of (a) 2×10^{-4} M Co(III) in the base solution (0.1 M NH_3, 0.1 M NH_4Cl, and 0.1 M KCl, pH 9.4) and (b—e) horse cyt-c at four different concentrations in (a) at the dme. Figure 2 shows cyclic voltammograms of (a) 2×10^{-4} M Co-(III) in the base solution and (b and c) 10^{-6} M horse cyt-c in (a) at two exposure times at the hmde. Detailed analysis of the polarographic and voltammetric

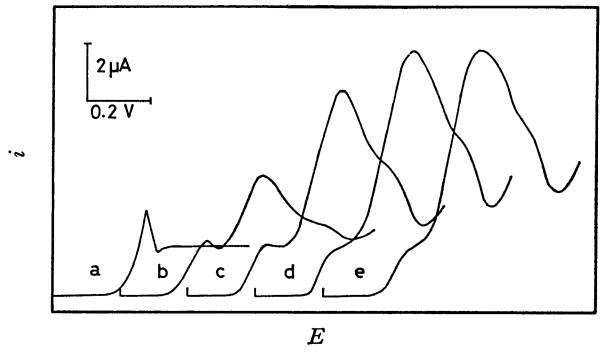

Fig. 1. D.c. polarograms of (a) 2.0×10^{-4} M Co(III) in the base solution and of horse cyt-c at (b) 4.0×10^{-7}, (c) 2.0×10^{-6}, (d) 4.0×10^{-6}, and (e) 5.7×10^{-6} M in (a). Polarograms are recorded from −0.9 V.

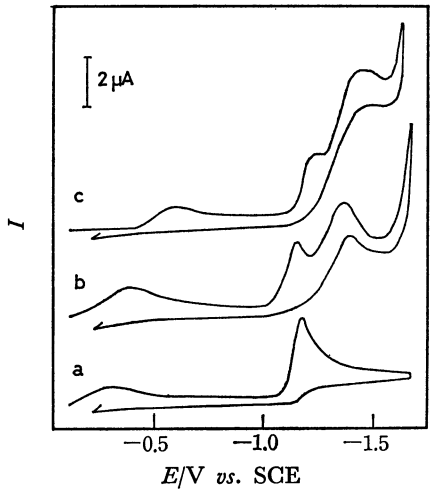

Fig. 2. Cyclic voltammograms of (a) 2.0×10^{-4} M Co(III) in the base solution and of (b and c) 1.0×10^{-6} M horse cyt-c in (a) exposuretim e: 4 s and 64 s, respectively, at the hmde.

behavior of the Brdička current produced by horse cyt-c at the dme and hmde has shown[10] that the dependence of the Brdička current-intensity at a given potential on various factors such as concentrations of the protein and Co(III), exposure time at hmde and drop time at dme is the same as that of the Brdička current produced by SS- and/or SH-containing proteins;[10,11] also that the Brdička current, i_B, at lower concentrations of Co(III), lower than 2×10^{-4} M in this case, can be expressed by

$$i_B = AFk_B\Gamma f_{Co} \quad (1)$$

where A is the electrode surface area, F Faraday constant, k_B a constant representing the Brdička current-activity of the protein studied, Γ the surface concentration of the protein adsorbed on the electrode surface, and f_{Co} the flux of cobalt ion at the electrode surface. It has also been shown[10,11] that the adsorption of protein on the mercury electrode surface at −1.4 V is controlled by diffusion and that Γ can be estimated by Koryta's equation.[12] The cobalt ion flux at the dme is given by Ilkovič theory. Thus the k_B value of horse cyt-c was determined by applying Eq. 1 to the Brdička current produced by horse cyt-c at −1.4 V, the diffusion coefficients of horse cyt-c and Co(III) being taken as 1.3×10^{-6} cm^2/s[13] and 7.5×10^{-6} cm^2/s,[11] respectively. The result is given in Table 1.

Figure 3 shows the temperature dependence of the polarograms of Brdička current produced by horse cyt-c from 10 °C to 25 °C. The current intensity at its maximum height (at −1.35 to −1.4 V) increased with rise in temperature. In contrast, it decreased at its shoulder at −1.5 V. The behavior is similar to that of SS- and/or SH-containing proteins.[10,14]

Brdička Current of Horse Cyt-c in the Presence of Other Metal Ions. In the presence of Co(II), cobaltous chloride, in place of Co(III), horse cyt-c gave a characteristic catalytic current practically the same in shape but slightly smaller in height than the catalytic current produced by horse cyt-c in the presence of Co(III). In the presence of Ni(II), nickel chloride,

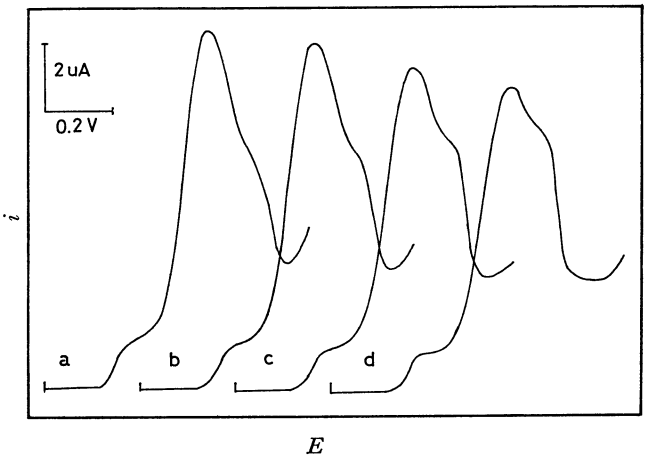

Fig. 3. Temperature dependence of d.c. polarograms of Brdička current of 4.0×10^{-6} M horse cyt-c in the base solution containing 2.0×10^{-4} M Co(III); temperature: (a) 25, (b) 20, (c) 15, and (d) 10 °C. Polarogramsa re recorded from −0.9 V.

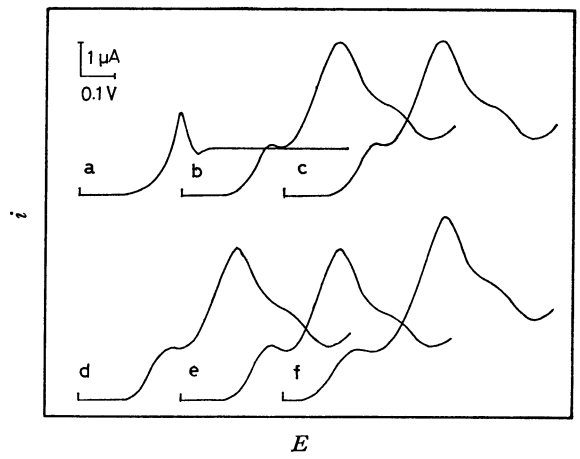

Fig. 4. D.c. polarograms of (a) 2.0×10^{-4} M Co(III) in the base solution and of 4.0×10^{-7} M cyt-c from (b) horse, (c) chicken, (d) tuna, (e) *C. krusei*, and (f) *S. cerevisiae* in (a). Polarograms are recorded from -0.9 V.

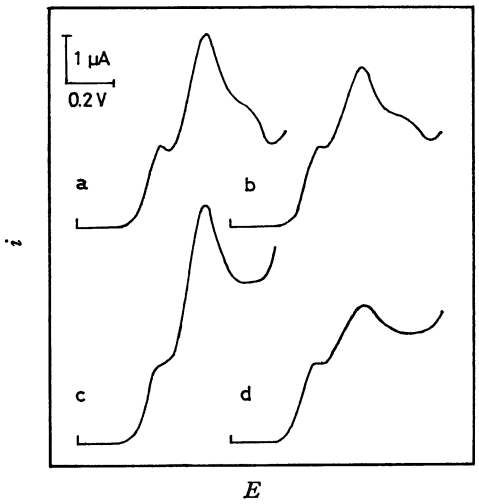

Fig. 5. D.c. polarograms of (a) native horse cyt-c, (b) CM-Cyt-c, (c) Co-cyt-c, and (d) porphyrin cyt-c each at 4.0×10^{-7} M in the base solution containing 2.0×10^{-4} M Co(III). Polarograms are recorded from -0.8 V.

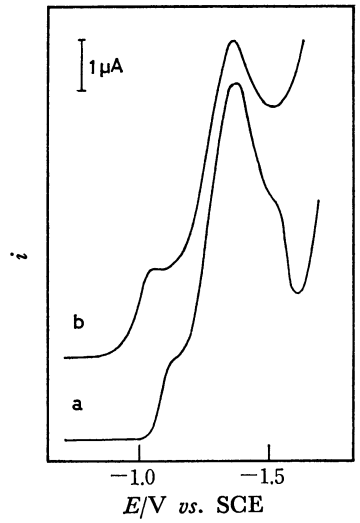

Fig. 6. D.c. polarograms of (a) 4.0×10^{-6} M native horse cyt-c and (b) 4.0×10^{-6} M apo-cyt-c in the base solution containing 2.0×10^{-4} M Co(III).

also catalytic current, though rather small, was produced. No catalytic current was observed when Co(III) was replaced by Cu(II), cupric nitrate, Zn(II), zinc chloride, and Mn(II), manganese chloride, each of 2×10^{-4} M.

Brdička Current of Cytochromes c from Other Sources. Figure 4 shows d.c. polarograms of cyt-c from five different sources at 4×10^{-7} M in a base soution containing 2×10^{-4} M Co(III). Chicken cyt-c and tuna cyt-c (Fig. 4, c and d) gave polarograms practically the same in both shape and height as horse cyt-c (Fig. 4b). *C. krusei* cyt-c also gave polarograms practically the same as horse cyt-c (Fig. 4e). At higher concentrations ($>2\times10^{-6}$ M), however, polarograms of *C. krusei* cyt-c were appreciably smaller in height as compared with those of horse cyt-c. *S. cerevisiae* cyt-c (Fig. 4f) gave polarograms which were different in shape and height from the four cytochromes mentioned above. The difference should be attributed to the existence of a free thiol group due to another cysteine residue.[15] k_B-values of these proteins determined by applying Eq. 1 to their Brdička currents at -1.4 V are given in Table 1.

Brdička Current of Modified Horse Heart Cytochromes. Figure 5 shows d.c. polarograms produced by (a) native horse cyt-c, (b) CM-cyt-c, (c) Co-cyt-c, and (d) porphyrin cyt-c each of 4×10^{-7} M in a base solution containing 2×10^{-4} M Co(III). The Brdička current of CM-cyt-c was very similar in shape to but smaller in height than that of native horse cyt-c. The Brdička currents of Co-cyt-c and porphyrin cyt-c differed more or less in shape and height from that of native horse cyt-c. Brdička currents of these modified proteins also have been found to follow Eq. 1. Their k_B values at -1.4 V are summarized in Table 1.

Apo-cyt-c gave Brdička current characteristic of denatured SS- and/or SH-containing proteins[10] (Fig. 6).

Brdička Current of D. vulgalis Cyt-c_3. Figure 7 and 8 show d.c. polarograms of *D. vulgaris* cyt-c_3 at four different concentrations in a base soution containing 2×10^{-4} M Co(III) at the dme and voltammograms of 4×10^{-7} M *D. vulgaris* cyt-c_3 at two exposure times at the hmde, respectively. Voltammograms of native cyt-c are also shown in Fig. 8. At lower surface concentration of adsorbed protein, *i.e.* at low bulk concentration with the dme or at smaller exposure time with the hmde, the polarograms (Fig. 7) and voltammograms (Fig. 8) of *D. vulgaris* cyt-c_3 were very similar in shape to but about double in height as compared with those of horse cyt-c. k_B-value of *D. vulgaris* cyt-c_3 determined at low protein concentrations at -1.4 V is given in Table 1. At high surface concentration of adsorbed protein, however, appreciable difference was observed especially at negative potential region (Figs. 1, 7, and 8).

Discussion

The polarograms of the Brdička current produced by Brdička current-active proteins are characteristic

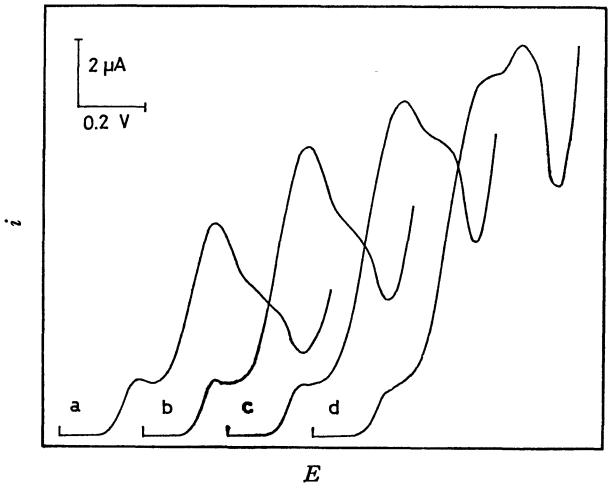

Fig. 7. D.c. polarograms of Brdička current of *D. vulgaris* cyt-c_3 at (a) 5.7×10^{-7}, (b) 1.7×10^{-6}, (c) 3.6×10^{-6}, and (d) 6.4×10^{-6} M in the base solution containing 2.0×10^{-4} M Co(III). Polarograms are recorded from −0.9 V.

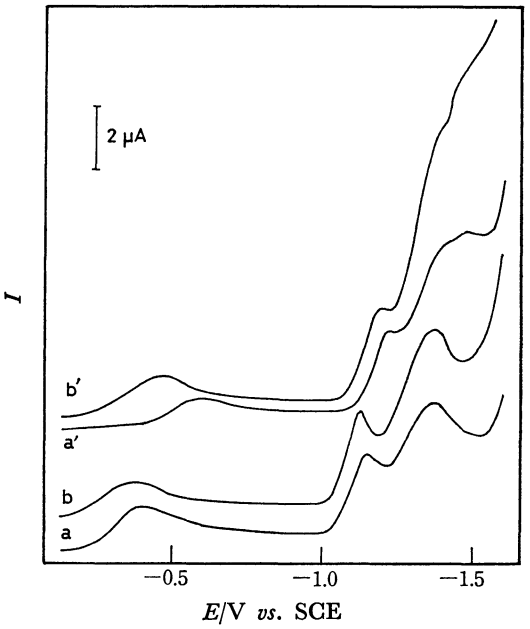

Fig. 8. Voltage-sweep voltammograms of (a and a') 4.0×10^{-7} M horse cyt-c at exposure time: 30 and 60 s, respectively and of (b and b') 4.0×10^{-7} M *D. vulgaris* cyt-c_3 at exposure time: 30 and 60s, respectively in the base solution containing 2.0×10^{-4} M Co(III) at the hmde.

of the proteins examined under specified conditions. Equation 1 predicts that the Brdička current is controlled by two factors, k_B and Γ. At and near −1.4 V the proteins we studied are very strongly adsorbed on the mercury electrode surface. At low bulk concentrations of the proteins, Γ at the dme surface can be evaluated by Koryta's equation, $\Gamma=(6/7)(D_p t_d/(3\pi/7))^{1/2}c_p$ *i.e.* as a function of the bulk concentration, c_p,

Table 1. Polarographic catalytic activities at −1.4 V of cytochromes c at pH 9.4

Protein	n_H	n_S	k_B / 10^{12} cm^2 mol^{-1}	6th ligand
Cytochrome c				
horse	1	0	4.2	Met[15]
chicken	1	0	4.2	Met
tuna	1	0	4.2	Met
C. krusei	1	0	4.2	Met
S. cerevisiae	1	1	5.8	Met
Modified cytochrome (horse)				
CM-cyt-c	1	0	2.3	Lys[19]
Co-cyt-c	1	0	4.7	Met[21]
porphyrin cyt-c	1	0	1.4	—
apo-cyt-c	0	2	2.2	—
Cytochrome c_3				
D. vurgaris	4	0	8.8	Lys[22] or His[23]

and the diffusion constant, D_p, of the protein and the age of a mercury drop, t_d, at the dme. With increasing negative potential, however, the proteins become less strongly adsorbed on mercury electrode surface and Γ can no longer be evaluated by Koryta's equation. Evaluation of Γ, which is now a function also of the electrode potential, becomes very difficult.[10] In the following, quantitative analysis of the Brdička current-activities of the proteins is limited to k_B-values at −1.4 V, where Koryta's equation is valid to evaluate Γ. It is noted, however, the potential dependence of Γ and that of k_B are responsible for determining the polarographic wave-form of Brdička current characteristic of the proteins examined, especially at negative potential region.

k_B(−1.4 V)-values of various proteins are given in Table 1, in which n_H and n_S are the numbers of heme groups and SH (plus $2\times$SS) groups, respectively, in one protein molecule. D.c. polarograms (Fig. 4) and k_B(−1.4 V)-values of horse, chicken, and tuna cyt-c indicate that the Brdička current-activities of these mitochondrial cytochromes are the same in spite of a difference in amino acid composition among these proteins. This indicates that the Brdička current-activity with these cytochromes is primarilly due to the heme group in their molecules.

C. krusei cyt-c also has the same activity as that of mitochondrial cytochromes at lower surface concentration of the adsorbed proteins (Fig. 4 and Table 1). At high surface concentration, however, a difference in Brdička current-activity was observed between *C. krusei* cyt-c and the mitochondrial cytochromes. This might be due to the fact[15] that the amino acid composition of *C. krusei* cyt-c differs from that of mitochondrial cytochromes and is characterized by a high content of proline. When proteins are adsorbed on mercury electrode surface, their conformational states change more or less.[16,17] The conformational state of protein adsorbed on water-air interface is highly dependent on the concentration of protein in the bulk of solution, from which the adsorption of proteins takes place.[18]

The difference in amino acid composition between *C. krusei* cyt-c and mitochondrial cytochromes c may cause a difference in the conformation of the proteins adsorbed on mercury electrode surface. The Brdička current can be interpreted to be due to the catalytic action of Co(0)-protein complex formed on electrode surface. The k_B-value depends on the intrinsic catalytic activity and stability of the Co(0)-protein complex, which are determined by the conformational state of protein adsorbed on the electrode surface.

The Brdička current-activity of *S. cerevisiae* cyt-c should be interpreted as the sum of the activity of a heme group and that of an SH group. The average $k_B(-1.4\ V)/n_S$ value of five SS- and/or SH-containing proteins (bovine serum albumin, lysozyme, ribonuclease, trypsin, and pepsin) was determined to be $1.4\pm0.2\times10^{12}$ cm²/mol.[10] Thus $k_B(-1.4\ V)=5.8\times10^{12}$ cm²/mol of *S. cerevisiae* cyt-c can be interpreted as the sum of $k_B(-1.4\ V)=4.2\times10^{12}$ cm²/mol of the heme group and $k_B(-1.4\ V)=1.6\times10^{12}$ cm²/mol of the SH group.

In the presence of adsorbed SS- and/or SH-containing protein on the electrode surface the reduction step Co(II) to Co(0) shifts positively by 0.2 V.[11,14] An interesting feature of d.c. polarograms and voltammograms of the Brdička current of horse cyt-c is that the reduction potential of Co(II) to Co(0) is not or only slightly (less than ±30 mV) affected by the presence of the adsorbed protein on electrode surface. When SS and/or SH groups coexist, as in the case of *S. cerevisiae* cyt-c (Fig. 4), the reduction step Co(II) to Co(0) shifts a great deal to positive potential (Fig. 6). The results indicate that the Brdička current produced by the cytochromes is due to the heme group in them.

When the heme group of horse cyt-c was modified, the Brdička current-activities of the proteins were drastically changed as expected from the above conclusion. The removal of the central metal atom from the heme reduced the activity to about one-third and also changed the shape of the Brdička current. Replacement of the sixth ligand of the heme iron from Met to Lys[19] also reduced the activity to almost one-half of the original value. At the potential where Brdička current is produced ferricytochrome c is reduced to ferrocytochrome c on electrode surface. This was confirmed by potential-controlled electrolysis of horse ferricytochrome c at −1.4 V on mercury pool electrode, the electrolyzed solution showing an absorption spectrum characteristic of ferrocytochrome c.[4]

The sixth heme ligand of ferricytochrome c can change from Met to Lys, depending on the proton dissociation of ε-amino group of lysine with $pK=9.3$.[15] Upon reducing ferricytochrome c with dithionite or hydrated electron[15,20] in a basic solution (pH 11) a ferro-form having lysine as the sixth ligand is produced transiently, with a subsequent relaxation step to Met-liganded ferrocytochrome c within several hundred milliseconds. We may thus conclude that the Met-liganded ferrocytochrome c plays a dominant role in producing the Brdička current of native cytochromes in ammoniacal buffer of pH 9.4. Replacement of the central metal atom from iron to cobalt resulted in a slight increase in the Brdička current-activity of cytochrome. The sixth ligand is methionine for both cobalti- and cobalto-forms of Co-cyt-c.[21]

The fact that $k_B(-1.4\ V)$-value of CM-cyt-c is about one-half of $k_B(-1.4\ V)$-value of native cytochromes is of interest in view of the result that the $k_B(-1.4\ V)$-value of *D. vulgaris* cyt-c_3, 8.8×10^{12} cm²/mol, is approximately equal to $4\times k_B(-1.4\ V)$-value of CM-cyt-c, $4\times2.3\times10^{12}$ cm²/mol, indicating that the sixth ligands of four hemes of *D. vulgaris* cyt-c_3 are lysine. This is in line with the proposed sixth ligands of the four hemes on the basis of laser raman spectroscopy,[22] though a proposed His-liganded cytochrome c_3[23] is not excluded since the polarographic effect of replacement of the sixth ligand from Met to His is not clear at present. The observed difference between polarograms and voltammograms of cyt-c_3 and cyt-c at higher concentrations should be interpreted in a similar way to that discussed above on Brdička current of *C. krusei* cyt-c.

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