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LIQUID CHROMATOGRAPHY–ELECTROCHEMICAL DETECTION OF FERRO- AND FERRICYTOCHROME *c* AT A CHEMICALLY MODIFIED GOLD ELECTRODE

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SUMMARY

Chemically modified electrodes, constructed by adsorption of 4,4′-dithiodipyridine onto a polyvinylferrocene-treated gold surface, were employed for the amperometric detection of cytochrome *c* following size-exclusion chromatography. The electrode response was nearly reversible, permitting quantitation both of the ferro-form of the protein at +0.15 V *vs.* Ag/AgCl and of the ferri-form at −0.15 V. The limit of detection for the reduced species was 3 pmol injected, and the response was linear over three orders of magnitude. Using the chemically modified electrode approach, cytochrome *c* monitoring was sufficiently selective that the compound could be determined in human plasma pretreated only by dilution and particulate filtration.

INTRODUCTION

Despite the fact that numerous biological macromolecules contain sites that are readily oxidized or reduced by chemical redox agents, electrochemically based analysis approaches have seldom been successfully employed for the direct detection and quantitation of these species. Large biomolecules have, of course, been frequently utilized indirectly in electroanalysis as, for example, in enzyme electrodes where the low-molecular-weight substrates or reaction products of immobilized enzyme systems are monitored at nearby potentiometric or amperometric electrodes. But the macromolecules themselves generally exhibit extremely poor electron transfer behavior at conventional electrodes. A few rather specialized electrode materials have been shown to give long-lived quasi-reversible response in some cases —notably, tin-doped indium oxide[1–3], fluorine-doped tin oxide[3], and edge-graphite[4] for cytochrome *c* and ruthenium oxide[5] for cytochrome *c*, azurin, rubredoxin, ferredoxin, and plastocyanin. However, these exceptions are few in number; and, even with the application of comparatively high potentials, most large molecules are not electrolyzed favorably enough to permit their direct detection to be a practical possibility. This result is unfortunate because many of the analytical capabilities usually attainable with electrochemical techniques would seem to make these methodologies ideally suited for trace level determination of various protein and nucleic acid analytes.

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One technique which might be particularly useful in these types of analyses is liquid chromatography with electrochemical detection (LC–ED). Over the past decade, this approach has met with considerable success in the determination of catechols and other easily oxidized low-molecular-weight biomolecules for which, in favorable cases, pmole quantities can be assayed in complex physiological samples with relatively simple procedures and economical instrumentation[6]. Obviously, in order for similar LC–ED advantages to be realized for significantly larger components such as an enzyme itself, some means of improving electrode response toward that species must be formulated so that its electrode reaction occurs facilely at a potential not far removed from its thermodynamic value.

A promising approach to this problem involves the use of chemically modified electrodes (CMEs) possessing surfaces specifically tailored to provide effective interaction with the macromolecule of interest at its electroactive site. Thus far, not many CMEs have been successfully constructed and employed for this purpose, the primary example reported to date being the family of pyridine-modified gold electrodes at which the redox protein cytochrome *c* (molecular weight = 12 384) exhibits nearly reversible behavior [7–11]. Despite thorough characterization, these electrodes have not yet, to our knowledge, been utilized for cytochrome *c* analysis and, specifically, for LC–ED. In principle, however, if sufficiently stable under LC–ED conditions, such CMEs —and analogous ones for other biomolecules— should have immediate application in numerous areas of bioanalysis.

In this work, we have attempted to demonstrate the analytical potential of CME-based LC–ED for redox-active biological macromolecules. To accomplish this objective in a straightforward manner, cytochrome *c* was selected as a model analyte. This system was chosen, not so much because of the overwhelming importance of the cytochrome *c* assay, but primarily because of the extensive body of work already in existence for cytochrome *c* at both modified and unmodified electrodes, much of which has been the subject of recent review[3,11]. Of particular concern here are the performance of this type of CME under LC–ED conditions and its potential advantages with respect to commonly employed UV–VIS spectrometric detection approaches. The specific electrochemical and chromatographic methodologies employed relate only to the cytochrome *c* assay system. But the conclusions can, we believe, be generally extended to possible LC–ED of numerous additional biological macromolecules.

EXPERIMENTAL

Reagents

4,4′-Dithiodipyridine, the cytochromes *c* (Type VI, from horse heart; Type V, from bovine heart; Type XVIII, from dog heart; Type X, from chicken heart; and Type XI, from tuna heart), catalase (from bovine liver), human hemoglobin, and myoglobin (Type II, from sperm whale skeletal muscle) were all obtained from Sigma. Unless otherwise specified, the cytochrome *c* used in a given experiment was the horse heart variety, Type VI which nominally was 96% pure and contained up to 10% in the reduced form. Ferrocytochrome *c* in the fully reduced state was obtained by treating the commercially obtained protein with an excess of sodium dithionite; a fully oxidized sample was obtained by treatment with potassium ferricyanide. Poly-

vinylferrocene was obtained from Polysciences. Pooled human plasma was purchased from the American Red Cross Blood Services, Louisville, KY, U.S.A. region. All reagents including cytochrome *c* were used as received without further purification.

Working electrodes

The working electrode was a Bioanalytical Systems (W. Lafayette, IN, U.S.A.) Model TL-6 gold electrode. The active surface consisted of a 3-mm diameter gold disc housed in a Kel-F block. The electrode was used in either of two forms, unmodified or modified with 4,4′-dithiodipyridine. An "unmodified" surface was generated by soaking the gold disc in concentrated nitric acid and/or by polishing with alumina. Further cleaning was accomplished by immersing the electrode in degassed pH 6.8 buffer and polarizing it for 10 min at +0.9 V *vs.* Ag/AgCl; after each polarization, the electrode was cycled between +0.55 V and −0.30 V until the background decreased to a stable level. Five repetitions were employed to obtain an unmodified surface that showed no significant background improvement upon further treatment. The electrode modification was carried out on a freshly polished surface, closely following the procedure of Taniguchi *et al.*[10]. One major change in the modification sequence was the addition of a pretreatment step which increased the response and durability of the subsequently modified surface. The pretreatment consisted of placing a thin layer of polyvinylferrocene onto the gold surface by first dip-coating the electrode two to three times in a 2 mg/ml solution of the polymer in dichloromethane and then immersing it in concentrated nitric acid for approximately 1 min. The lengths of time allotted for each of these steps depended on the thickness desired for the polymer layer, with best CME activity obtained when a relatively thin coating was achieved (as in Fig. 1). Next, the electrode was thoroughly rinsed with deionized water, dried, and placed for 5 min in a 9 m*M* solution of 4,4′-dithiodipyridine in dichloromethane. After rinsing, the CME was ready for use.

Apparatus

Cyclic voltammetry was performed with a Bioanalytical Systems Model CV-1B potentiostat and a Hewlett-Packard Model 7035B *x–y* recorder. A gold working electrode (with or without modification), an Ag/AgCl reference, and a platinum wire auxiliary electrode were used for all experiments.

Flow injection analysis and high-performance liquid chromatography (HPLC) were performed with a Waters Assoc. Model M-45 pump, a Rheodyne (Berkeley, CA, U.S.A.) Model 7125 injector with a 20-μl sample loop, a Bioanalytical Systems Model LC-3 amperometric detector and Model TL-6 thin-layer cell, and a Fisher Series 5000 recorder. An Ag/AgCl electrode served as the reference. In HPLC, a Synchrom (Linden, IN, U.S.A.) GPC-100 size-exclusion column was employed; the 250 mm × 4.6 mm I.D. column contained 5-μm glycerolpropylsilane-bonded silica particles with 100-Å pores. The mobile phase used in all HPLC and electrochemical experiments was a pH 6.8 phosphate buffer (20 m*M* KH_2PO_4, 26 m*M* Na_2HPO_4) containing 100 m*M* $NaClO_4$.

Absorbance spectra were recorded on a Varian Cary 219 spectrophotometer. Absorptivity values were determined at 550 nm on standard cytochrome *c* solutions either fully reduced with $Na_2S_2O_4$ or fully oxidized with $K_3Fe(CN)_6$. The values determined were 30.0 mM^{-1} cm^{-1} and 8.4 mM^{-1} cm^{-1} respectively. These agree well with the absorptivities previously reported for the two redox states of the enzyme[12].

RESULTS AND DISCUSSION

CME performance

The electrochemical behavior of cytochrome *c* has previously been examined at a variety of electrode surfaces including gold, platinum, mercury, and tin and indium oxide semiconductors[3]. The response obtained ranged from reversible to irreversible, with the rate of electron transfer depending, especially for the metal electrodes, on the state of "cleanliness" of the particular surface employed. For example, at a gold electrode, observation of a faradaic cytochrome *c* signal is possible only after special pretreatment involving heating in a hydrogen flame or an air plasma or, less satisfactorily, immersion in strong acid or sonication in detergent solution. Failure to pretreat the electrode by these procedures resulted in the absence of any response for the protein. Furthermore, even with pretreatment, usage of the electrode or even just exposure of the pretreated surface to buffer solution produced a rapid decrease in cytochrome *c* response presumably due to the adsorption of trace organic impurities or other solution components. Clearly, the instability of metallic electrodes renders their use in quantitative analytical applications impossible. While the cytochrome *c* response at tin and indium oxide electrodes does not appear to be quite so unstable, it is still far from ideal.

Several electrode modification strategies have been reported to be effective for cytochrome *c*, the most attractive for the purposes of this work consisting of the family of pyridine-modified gold electrodes first reported by Eddowes and Hill[7]. Initially, 4,4′-bipyridine and, subsequently, a large group of adsorbable anionic or basic modifiers have been shown to permit the electro-oxidation–reduction of the protein to take place at nearly its thermodynamic potential, +0.065 V *vs.* Ag/AgCl[13]. Apparently, interaction between the modifier molecules and positively charged lysine residues on cytochrome *c* results in the formation of a transient complex in which the protein's barely exposed heme crevice and the electrode surface are favorably oriented for electron transfer[14,15]. Consistent with this hypothesis is the observation that the activity of the CME toward cytochrome *c* is lost under acidic conditions where the pyridine groups are protonated. The CMEs involved are usually formed by simple dip-coating procedures and, depending on the strength of the modifier adsorption, can give very stable activity in conventional cyclic voltammetry studies.

Of the various modifying agents that have been examined for promotion of the cytochrome *c* electrode process, 4,4′-dithiodipyridine seemed best suited for the analytical and LC–ED applications of interest in this study. This modifier, suggested and initially characterized by Taniguchi *et al.*[9,10], is a highly effective promoter (heterogeneous rate constant $k_s = 6 \cdot 10^{-3}$ cm/s) and also adsorbs particularly strongly at the gold surface. Thus, 4,4′-dithiodipyridine was the modifier selected and subsequently employed here. No other modifiers were investigated; but it is possible, of course, that other cytochrome *c* promoters might give similar or even superior analytical performance.

One significant difference in CME preparation compared to the method employed by Taniguchi *et al.*[10] was found to be useful for this work. As detailed in the Experimental section, a thin coating of polyvinylferrocene was first placed on the bare gold surface and exposed to concentrated nitric acid prior to adsorption of the 4,4′-dithiodipyridine. Electrodes pretreated by this relatively simple procedure were

consistently found to exhibit greater cytochrome *c* response, slower loss of response, and decreased background current. The reasons for these improvements have not yet been determined.

Cyclic voltammograms (CVs) obtained at both bare and dithiodipyridine-modified gold electrodes for a pH 6.8 solution containing horse heart cytochrome *c* are shown in Fig. 1. Also shown for comparison is the CV for the CME immersed in blank buffer only. Cytochrome *c* response, observed only at the modified surface, consisted of a nearly reversible pair of waves at +0.06 V and −0.02 V *vs.* Ag/AgCl. In addition, a set of redox waves corresponding to the oxidation and reduction of the ferrocene moiety from the residual polyvinylferrocene coating also occurred at more positive potentials both in the presence and absence of cytochrome *c*. As expected for a simple quasi-reversible electron transfer, the cytochrome *c* currents were proportional to the protein concentration employed and to the square root of the potential scan rate (up to 400 mV/s). No apparent response toward the protein was seen at the unmodified electrode. This is not surprising as the special pretreatment procedures required for effective cleaning of the bare gold surface were not employed here. The CV behavior observed is completely consistent with that previously reported for similarly modified and unmodified gold electrodes[10,11].

It is relevant to emphasize that, throughout this work, no purification procedures were applied to the commercially purchased cytochrome *c* samples employed. Although the presence of impurities in such samples might exercise an adverse effect on electrode response (especially for the bare gold electrode), it was felt that, for an

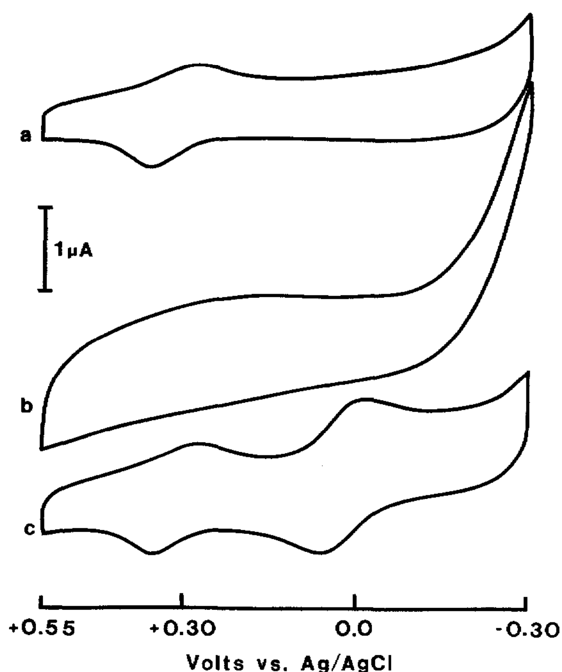

Fig. 1. Cyclic voltammograms of (a) CME in pH 6.8 phosphate buffer, (b) clean gold electrode in 0.12 m*M* horse heart cytochrome *c* in pH 6.8 buffer, and (c) CME in same solution as for (b). Scan rate: 100 mV/s.

analytically oriented study, it would not be realistic to exclude rigorously the occurrence of such contaminants. Thus, it is possible that, in some instances, electrode performance reported here is not as "good" as that reported previously in related studies of cytochrome *c* where extensive clean-up procedures were employed.

Two specific aspects of the dithiodipyridine-modified electrode response were of particular interest for anticipated analytically oriented applications. These were the CME's capability for sensing each of the redox forms of cytochrome *c* at extremely modest operating potentials and the stability of the CME's activity over an extended period of use. The potentials observed for the CME-promoted electrode process agree well with the thermodynamic potential reported for the hemoprotein[13]. More importantly for analysis, and especially for the flow injection and LC–ED applications of interest in this work, the oxidation and reduction occurred at low potentials in a relatively noise-free region optimum for sensitive and selective detection. Further, the CME continued to exhibit virtually undiminished response to cytochrome *c* even over the course of several hours of cycling over the indicated potential range. As has been reported earlier, however, the activity of the CME was inhibited in acidic solutions in which the attached pyridine groups are protonated and thus not able to interact productively as required with the protein's lysine residues[11].

Accordingly, we sought to characterize the performance of the dithiodipyridine CME for cytochrome *c* quantitation in flow and LC–ED systems. Representative chromatograms obtained at both the modified and unmodified gold electrode are provided in Fig. 2. The chromatographic method utilized consisted of a size exclusion

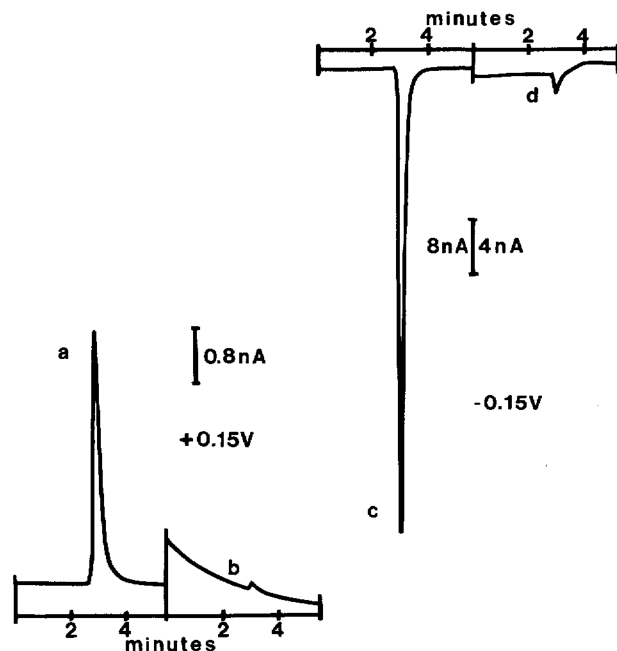

Fig. 2. Chromatograms of horse heart cytochrome *c* at +0.15 V (a, b) and −0.15 V *vs.* Ag/AgCl (c, d). Curves (a) and (c) were obtained at the 4,4′-dithiodipyridine CME; (b) and (d) were obtained at a clean gold electrode. Quantity injected, 2.4 nmol; mobile phase, pH 6.8 phosphate buffer; flow-rate, 1.0 ml/min.

separation similar to that typically employed for isolation of the protein from common physiological matrices[16]. The sample examined was prepared from commercially available cytochrome *c* and contained the ferri- and ferro-forms in a nominal 9:1 proportion; therefore, depending on the specifically applied potential, both reduction and oxidation responses were expected. In practice, this turned out to be the case. Operation at −0.15 V *vs.* Ag/AgCl produced a large (roughly 70 nA) cathodic peak in chromatogram *c* at a retention time of 3 min while detection of the identical sample at +0.15 V resulted in a 4-nA anodic peak in chromatogram a having essentially the same retention. Under the same experimental conditions, the unmodified electrodes yielded drastically smaller current signals (chromatograms d and b) for identical cytochrome *c* concentrations. (Increases in detector potential produced no increase in signal at the plain gold surface but did cause severe increases in the already substantial background current.)

Hydrodynamic voltammograms (HDVs) obtained for the modified electrode by measuring cytochrome *c* peak current as a function of potential for a series of individual injections are shown in Fig. 3. Curve a represents the HDV measured directly for the commercial (*i.e.*, mixed ferri-/ferro-) cytochrome *c* sample while curve b was obtained after the same sample had been reduced with sodium dithionite. As expected from the CV data, the overall shapes of the two HDVs were essentially the same, the principle difference being that the former exhibited both anodic and cathodic current components while the latter fully reduced sample naturally gave only an anodic response. Comparison of the current levels generated by each of the redox forms indicated that, of the two processes, the ferrocytochrome *c* oxidation gave somewhat more current than was obtained for reduction of the corresponding quantity of the ferri protein. Because this difference is appreciable (roughly 30%), accurate

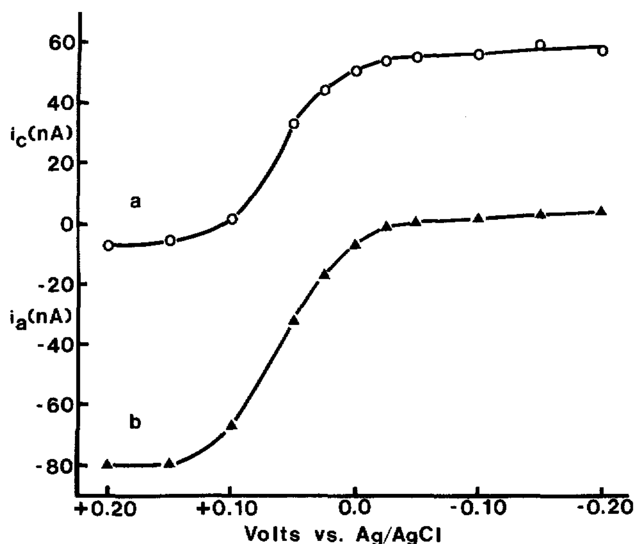

Fig. 3. Hydrodynamic voltammograms obtained at CME for (a) 0.80 nmol horse heart cytochrome *c* commercial sample and (b) same sample reduced with $Na_2S_2O_4$. Chromatographic conditions were the same as in Fig. 2.

quantitation by LC–ED will require the use of the correct redox form of the protein for calibration purposes.

On the basis of the HDVs, it was apparent that optimum potentials for LC–ED detection of the ferro- and ferricytochrome *c* should be roughly +0.15 V and −0.15 V, respectively. Under these conditions, the lower limit of detection (signal-to-noise ratio = 2) for the ferro protein was 3 pmol injected. The corresponding calibration curve, computed by least squares analysis of data for seven concentrations, was linear over at least three orders of magnitude (i = 65.0 nA/nmol $\cdot \chi -$ 0.18 nA where χ is the number of nmol of ferrocytochrome *c* injected; correlation coefficient = 0.99). Quantitation of the ferri protein generated by oxidation with ferricyanide could be performed in similar fashion but at −0.15 V. Detection limits so obtained were somewhat poorer because of incomplete chromatographic resolution of the protein from excess ferricyanide. Therefore, selection of different chromatographic conditions or of a different oxidizing agent is recommended for optimum ferricytochrome *c* detection. CME response at these potentials and flow conditions was extremely stable, decreasing typically by only 10% over the course of 18 h of continuous LC–ED operation. When stored with no potential applied, the electrode activity was not diminished at all even for much longer periods. Furthermore, because the time required for the background current to settle down to an acceptable level was only 5–10 min for the CMEs, simply removing it from the circuit when not in actual use presented no practical problem. Interestingly, the LC–ED background observed for the bare Au surface was much higher in magnitude than that for the CME and typically required 1–2 h to reach a stable level. This is consistent with previous findings that, when contaminants are removed from a gold electrode, the resulting hydrophilic surface exhibits high charging currents which slowly subside as it is recontaminated by organic impurities[3].

Applications

One readily apparent property of the dithiodipyridine CME is its ability to differentiate directly the ferro- and ferri-forms of cytochrome *c*. The most widely accepted procedure for this determination requires measuring the difference in absorbance at 550 nm between the cytochrome *c* sample of interest and a fully oxidized or reduced reference sample[17] or, in some cases, an unincubated, commercial reference. Alternately, some researchers have chosen to subtract the background absorbance at 540 nm from the sample absorbance at 550 nm and employ various absorptivity values[18]. The problem becomes significant in situations where the total cytochrome *c* concentration is low, where the cytochrome *c* is present in preponderantly one of the two oxidation states, or where spectral interferents are present.

None of these difficulties applies to the CME assay suggested here. Once a calibration has been established, a single injection is sufficient to specify absolutely the quantity of either redox form. Quantitation of the other oxidation state requires only a change in the detector potential and a second sample injection. In principle, such a determination could be performed without a chromatographic column by a simple flow injection approach as long as no other electroactive sample components are present; however, if other electroactive species are present in the sample, a prior chromatographic separation would, of course, be of some use. In this work, a qualitative comparison of the CME-LC–ED method with the difference absorbance ap-

proach was made by comparing results obtained on a commercial horse heart cytochrome *c* sample which was nominally 90% oxidized when purchased. In this test, the ferrocytochrome *c* content was estimated at only 4% by the absorbance method and 8% by the LC–ED procedure. While it is not possible to determine with certainty which assay was in fact the more accurate, the LC–ED approach was clearly the more straightforward to carry out and more capable of extension to substantially lower quantities of cytochrome *c*.

A second characteristic of the CME response which is of relevance for possible analytical usage is the low operating potential required. Thus, unlike absorbance measurements, CME-based assays should be quite selective toward cytochrome *c* and more readily adapted for use in analyses performed in complex sample matrices such as blood or other physiological media. While the determination of cytochrome *c* is not itself of widespread clinical importance, its assay is often used to quantitate physiological levels of other redox active biochemical species such as superoxide anion[19]. Since superoxide anion reduces ferricytochrome *c*, the oxidized protein is added in excess to a wide variety of assay samples; subsequently the level of ferrocytochrome *c* produced is determined and related to superoxide anion production[20–23]. In studies such as these, the critical quantity to be determined is not, of course, the total amount of cytochrome *c* present (which is ordinarily known) but the amount present in a particular redox state. Accordingly, Fig. 4 contains size-exclusion chromatograms obtained at appropriate potentials for human plasma containing cytochrome *c* added in either the reduced (curve a) or oxidized (curve b) form. In both cases, no significant interfering species were seen despite the fact that sample treatment consisted of simply diluting the plasma with buffer and filtering out particulates.

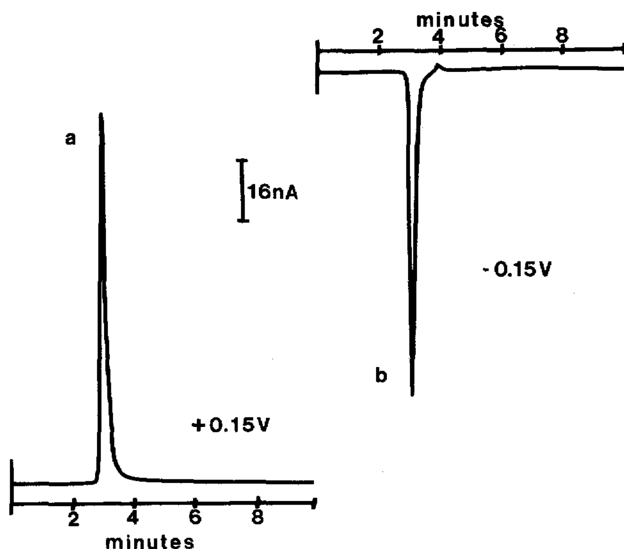

Fig. 4. Chromatograms obtained at CME for 1.6 nmol cytochrome *c* in 30% human plasma–pH 6.8 buffer (30:70). (a) $E = +0.15$ V *vs.* Ag/AgCl; cytochrome *c* was fully reduced with $Na_2S_2O_4$; (b) $E = -0.15$ V; cytochrome *c* sample was run as obtained in 90% oxidized state. Conditions were the same as in Fig. 2.

Finally, in an effort to evaluate the applicability of the dithiodipyridine CME for other possible analytes, its response toward some additional hemoproteins and cytochrome *c* variants was examined. The other hemoproteins considered, catalase, hemoglobin, and myoglobin, exhibited no significant response at the CME. This was not surprising considering the nature of the mechanism which has been suggested for the pyridine–horse heart cytochrome *c* interaction[7,11,14,15] and the drastically different structures of the other hemoproteins. However, the other cytochromes *c* tested (from bovine, dog, chicken, and tuna sources) all possess essentially the same structure as the horse heart variety examined above, and all produced essentially the same electrochemical behavior. Likewise, the chromatographic behavior of all the cytochrome *c* varieties was similar in terms of both retention characteristics and current response.

A detailed compilation of the LC–ED response of the cytochromes *c* is given in Table I, using the horse heart species as the standard of comparison. Total currents were computed for each of the different samples by summing the peak currents for chromatograms obtained at both oxidizing and reducing potentials; this was necessary to correct for differences in the ferro-/ferri- content of the various commercially obtained samples. Furthermore, because the solutions analyzed were of slightly different concentration, current values expected for reactivity identical to that of the horse heart species were computed by normalizing the horse heart response by a factor proportional to the concentration difference. Once these corrections were applied, the current found for each cytochrome *c* was virtually the same in every case except for the tuna which produced only 64% of the expected response. Possibly, this decrease is attributable to the fact that tuna cytochrome *c* contains more differences (with respect to horse heart cytochrome *c*) in its amino acid sequence than do the other cytochromes *c* tested[24]. In addition, tuna cytochrome *c* contains only 16 lysine residues, three fewer than horse heart. Since it is specifically the lysine groups which are thought to be responsible for the interaction with the CME surface, this difference might also explain the diminished response toward tuna. While the missing lysine residues are not those closest physically to the exposed heme edge, they may nevertheless play some role in effectively orienting the molecule for electron transfer.

The dithiodipyridine-containing Au electrode developed by Taniguchi *et al.*[9]

TABLE I

DETERMINATION OF A VARIETY OF CYTOCHROMES *c*

	Total current (nA)	*Current expected (nA)**	*Current found (%)*	*No. of differences in amino acid sequence*,***	*No. of Lys residues***
Horse heart	24.6	24.6	100	–	19
Bovine	23.8	23.0	103	3	18
Dog	21.4	23.6	91	5	18
Chicken	19.5	19.5	100	11	18
Tuna	11.0	17.3	64	19	16

* Relative to horse heart cytochrome *c*.
** See ref. 15.

and Allen *et al.*[11] and employed in this work is one of the few available CMEs which have been shown to provide enhanced electrochemical activity toward important biological macromolecules. It is clear that, as a result of electrode modification, some of the principal analytical advantages of LC–ED —namely, sensitivity and selectivity— are able to be obtained for cytochrome *c*. Further, electrochemical detection also affords the unique capability to differentiate directly its two redox states. The surface modification employed here appears to be very specific in its response to the cytochrome *c* structure and thus provides an electrode which is activated only toward this limited set of analytes. Other CMEs yet to be devised may also exhibit a marked structural selectivity, or they might be more generally applicable in their mode of action. However, in either case, it is clear that these devices, possessing surface forms tailored specifically for their intended function, offer some distinct advantage in the electroanalysis and, in particular, the LC–ED of oxidizable and reducible biomolecules.

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