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ELECTROCHEMICAL REACTIONS OF HORSE HEART CYTOCHROME *c* AT GRAPHITE ELECTRODES

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The interaction of cytochrome *c* with a paraffin-wax-impregnated spectroscopic graphite electrode (WISGE) was studied in a medium consisting of 0.1 M potassium phosphate, pH 7.0, by means of differential pulse and cyclic voltammetry. Ferricytochrome *c* yields on voltammograms a single cathodic peak C around a potential of −0.3 V (vs. Ag/AgCl) and two anodic peaks A_I and A_{II} around the potentials of 0.66 and 0.89 V, respectively. Cathodic peak C corresponds to a catalytic reaction during which ferricytochrome *c* is reduced to ferrocytochrome *c*: ferricytochrome *c* is then regenerated by chemical oxidation of ferrocytochrome *c* by oxygen adsorbed at the WISGE surface. The first, more negative anodic peak A_I corresponds to anodic electrochemical odixation of tyrosine residues, whereas the second, more positive anodic peak (peak A_{II}) corresponds to an anodic reaction of haemin. Voltammetry at a WISGE may provide a valuable technique for obtaining data about cytochrome *c* properties on electrically charged surface.

1. Introduction

Horse heart cytochrome *c* is a relatively small protein which is composed of 103 amino acids, linked together in a single polypeptide chain wrapped around a haem [1]. In vivo cytochrome *c* is associated with the mitochondrial membrane system, where it functions as an electron-transfer carrier. A number of different experimental techniques have now been used to study the mechanisms by which this protein performs its biological function. While the properties of cytochrome *c* in solution are well known, its properties in a membrane situation are not so well understood. As it is difficult to study the latter properties directly under physiological conditions, experimental approaches are being sought that would make it possible to obtain basic data about the behaviour of cytochrome *c* in the mitochondrial membrane. Results of some studies indicate that various electrically charged surfaces, especially electrodes used in electrochemical analysis, are a suitable tool for studying this behaviour of cytochrome *c* (e.g., see refs. 2–6).

The investigation of interfacial properties of this protein at electrodes with the aid of methods of electrochemical analysis may provide new information, not only about redox properties of cytochrome *c*, but also about the conformation of the protein in the adsorbed state. The latter property of cytochrome *c* seems to be of particular interest because its conformation in the mitochondrial membrane system is expected to have many features in common with the conformation of cytochrome *c* adsorbed at an electrode/solution interface.

It is well known that voltammetry at mercury electrodes is applicable to the study of interfacial properties of proteins [7–9], including cytochrome

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c [4,6,10]. Quite recently, voltammetry at carbon electrodes has been applied to the study of electro-oxidation of tyrosine and tryptophan residues in some globular proteins [11–13]. The object of this report is to describe the interfacial behaviour of horse heart cytochrome *c* at a paraffin-wax-impregnated spectroscopic graphite electrode.

2. Materials and methods

Horse heart ferricytochrome *c* (type VI) from Sigma Chemical Co. was used without further purification. The absence of electroactive impurities was verified by a method described earlier [14]. Apocytochrome *c* was prepared by the method of Stellwagen and Rysavy [15]. Porphyrin cytochrome *c* was prepared and used in the same way as described by Ikeda et al. [16]. Chemically reduced cytochrome *c* was prepared by the addition of a few grains of sodium borohydride to 2 ml of 5×10^{-5} M ferricytochrome *c* in 0.1 M potassium phosphate, pH 7.0. Completeness of reduction ($\approx$ 90%) was estimated by observing the 550 nm band, which is typical of the reduced form of cytochrome *c*. Cytochrome c_3, and ferredoxin I and II, all from *Desulfovibrio desulfuricans* (Norway strain) and cytochrome *c*-553 from *Desulfovibrio vulgaris* (Hildenborough strain), were generous gifts of Dr. M. Bruschi, Laboratoire de Chimie Bactérienne du C.N.R.S., Marseilles. The latter three proteins were prepared and purified as previously described [17–19]. Metmyoglobin from sperm whale skeletal muscle was obtained from Sigma Chemical Co. Lysozyme (from hen egg white) and ribonuclease (from bovine pancreas) were obtained from Nutritional Biochemicals (OH) and Reanal (Hungary), respectively. Tobacco mosaic virus (TMV) protein was the generous gift of Ing. G. Ruttkay-Nedecký. Basic characteristics of proteins used in this work are specified in table 1.

4,4'-Bipyridyl was obtained from Fluka. Chemicals used for preparation of background electrolyte solutions were of analytical grade. Potassium phosphate (KH_2PO_4/K_2HPO_4) was used for voltammetric measurements at a concentration of 0.1 M and had a pH of 7.0.

The paraffin-wax-impregnated spectroscopic graphite electrode (WISGE) and pyrolytic graphite electrode (PGE) were prepared and used in the same way as described earlier [12,20]. Between runs, the WISGE and PGE were resurfaced by polishing the ends with emery polishing paper (Feuilles Papier Abrasif Emery Polishing, CEN 4/0). The WISGE and PGE had geometric areas of 7 and 13 mm^2, respectively.

Differential pulse (DP) voltammograms were obtained with a PAR Model 174A polarographic

Table 1

Some characteristics of the proteins used in this work

	Molecular weight	Number of amino acid residues/molecule			Number of haemin/molecule
		Tyrosine	Tryptophan	Cysteine	
Cytochrome *c*	12384	4	1	2	1
Cytochrome c_3	13000	1	0	8	4
Cytochrome *c*-553	9100	6	0	2	1
Myoglobin	17800	3	2	0	1
Ferredoxin I [a]	6000	0	0	16	0
Ferredoxin II [a]	6000	1	0	16	0
Ribonuclease	13683	6	0	8	0
Lysozyme	14400	3	6	8	0
TMV protein [a]	17500	4	3	1	0

[a] Characteristics relate to monomer subunits.

analyzer. All DP voltammograms were obtained with a pulse amplitude of 0.025 V and at a sweep rate of 0.005 V s^{-1}. The current sampling for DP voltammetry at the graphite electrodes was set with the drop-time control of the PAR Model 174A at 0.5 s. Cyclic voltammetry (CV) experiments were performed with a system consisting of a PAR Model 175 Universal Programmer and a PAR Model 173 Potentiostat equipped with a PAR Model 176 Converter. Voltammetric curves were recorded on a Sefram XY recorder. A Metrohm Ag/AgCl (satd. NaCl) electrode was used as reference electrode and a platinum wire as a counter electrode. Throughout this report, all potentials are given vs. an Ag/AgCl reference electrode. Unless otherwise stated, oxygen was purged from solutions by bubbling U-grade nitrogen for 30 min before recording the voltammogram.

The basic procedure for DP and cyclic voltammetry has been described previously [12,20]. However, the procedures described earlier were changed slightly in the following way. Once the graphite electrode was inserted into the test solution contained in the electrochemical cell, it was allowed to stand for approx. 10 s without an applied potential. Then, unless stated otherwise, the initial potential (E_i) was applied for a further 120 s, after which the voltammetric sweep was commenced. The electrochemical experiments were performed at 27°C. Other details of our measurements have been published elsewhere [11,12,20].

3. Results

3.1. Differential pulse voltammetry

The initial studies were carried out at the WISGE with deoxygenated solutions of ferricytochrome *c* at a concentration of 5×10^{-8} M. If the DP voltammogram was scanned from $E_i = 0.2$ V to negative potentials (using a cathodic pulse), ferricytochrome *c* yielded a single well defined peak C around − 0.3 V (fig. 1a). If the DP voltammogram was scanned from $E_i = 0.0$ V to positive potentials (using an anodic pulse), ferricytochrome *c* gave rise to two peaks, A_I and A_{II}, having peak potentials (E_p) of 0.66 and 0.89 V, respectively (fig. 1b). With the aim of identifying sites in the ferricytochrome *c* molecule responsible for the appearance of peaks C, A_I and A_{II}, DP voltammograms at the WISGE were also recorded for further proteins (figs. 1 and 2) and for chemically treated cytochrome *c* (not shown). All further haemin-containing proteins (table 1) studied in this work (i.e., cytochrome c_3, cytochrome *c*-553 and metmyoglobin) at a concentration of $2–5 \times 10^{-8}$ M yielded peak C. Ferredoxins, porphyrin cytochrome *c*, apocytochrome *c* and non-conjugated proteins (ribonuclease, lysozyme and TMV protein) did not yield this peak. 5×10^{-8} M cyto-

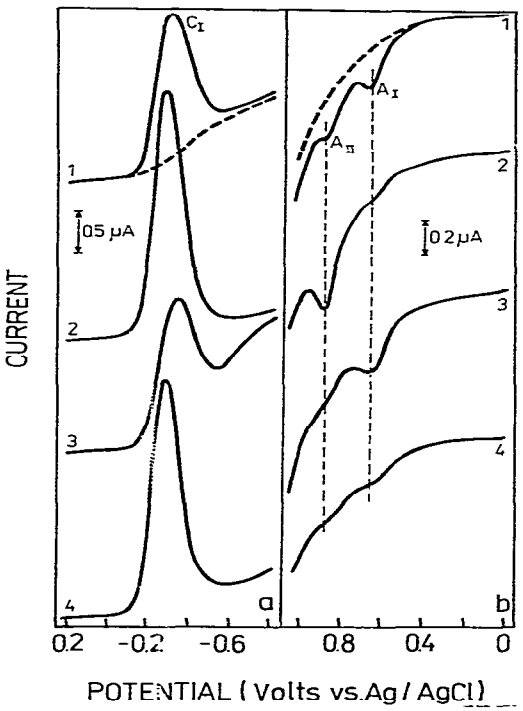

Fig. 1. DP voltammograms at the WISGE in 0.1 M potassium phosphate, pH 7.0, of 5×10^{-8} M cytochrome *c* (curves 1), 4.4×10^{-8} M cytochrome c_3 (curves 2), 2×10^{-8} M cytochrome *c*-553 (curves 3) and 2×10^{-8} M metmyoglobin (curves 4). (a) Initial potential was 0.2 V, cathodic potential sweep. (b) Initial potential was 0.0 V, anodic potential sweep. Waiting time at the initial potential was always 120 s. (- - - - -) Background electrolyte curve.

chrome *c* which was previously chemically reduced (completeness of reduction was about 90%) produced peak C, but its height was markedly lower than that of peak C of the untreated ferricytochrome *c*.

The anodic peak A_I was produced by apocytochrome *c*, porphyrin cytochrome *c*, chemically reduced cytochrome *c* and also by all native proteins studied except ferredoxin I. Anodic peak A_{II} was, however, produced only by haemin-containing proteins (fig. 1b) and by chemically reduced cytochrome *c*. Ferredoxins, apocytochrome *c* and ribonuclease gave rise to no futher anodic peak (except peak A_I). 1.4×10^{-5} M lysozyme and 5.7×10^{-6} M TMV protein yielded one more anodic peak at more positive potentials with an E_p of 0.82 V (fig. 2), i.e., with an E_p 70 mV more negative than that of peak A_{II} yielded by haemin-containing proteins (fig. 1b).

DP voltammograms of ferricytochrome *c* were also recorded at a PGE under conditions identical to those under which the experiments at the WISGE were performed (fig. 1a). Ferricytochrome *c* also yielded cathodic peak C at the PGE, but its height was about 20-times lower than that of peak C obtained at the WISGE (fig. 1a, curve 1).

The height of peaks C, A_I and A_{II} increased linearly at first with growing ferricytochrome *c* concentration (fig. 3). At higher ferricytochrome *c* concentrations these dependences tended to a limiting value. The height of peak C, after reaching the maximum value at a cytochrome *c* concentration of approx. $0.7–1.0 \times 10^{-7}$ M, was slightly lowered with a further increase in the protein concentration. The lower level of analytical utility of DP voltammetry at the WISGE for determination of ferricytochrome *c* is about 4×

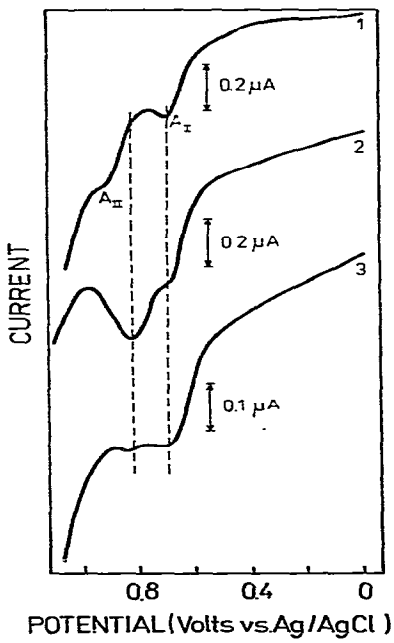

Fig. 2. DP voltammograms at the WISGE in 0.1 M potassium phosphate, pH 7.0, of 5×10^{-8} M cytochrome *c* (curve 1), 1.4×10^{-5} M lysozyme (curve 2), 5.7×10^{-6} M TMV protein (curve 3). Other conditions were the same as in fig. 1b.

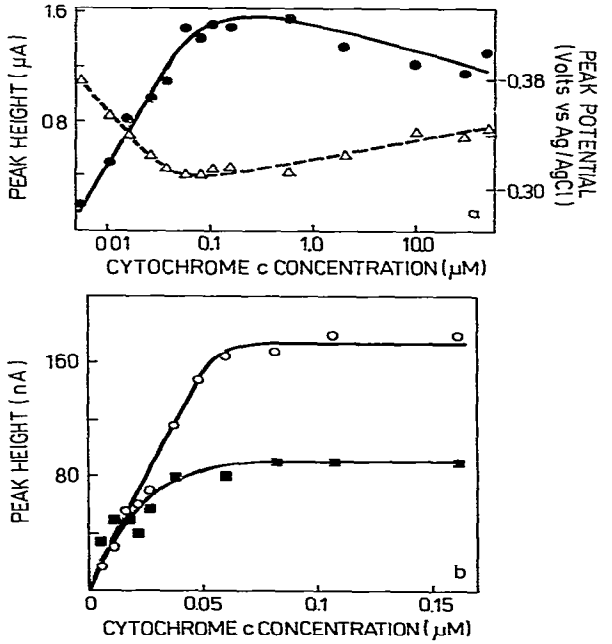

Fig. 3. (a) Variation of the DP voltammetric peak C height (●) and peak C potential (△) with concentration of cytochrome *c* at the WISGE. Other conditions were the same as in Fig. 1a. (b) Variation of the height of DP voltammetric peaks A_I (○) and A_{II} (■) with concentration of cytochrome *c* at the WISGE. Other conditions were the same as in fig. 1b.

10^{-9} M. E_p of peak C was shifted first to more positive values with growing ferricytochrome *c* concentration (fig. 3a). After reaching the maximum height of peak C a further increase in ferricytochrome *c* concentration led only to a slight shifting of the peak to more negative potentials. Peaks A_I and A_{II} of ferricytochrome *c* were shifted in the range of concentrations up to approx. 5×10^{-8} M to more positive potentials, approximately according to the equations:

$$E_p(A_I)\ (V) = 5.88 \times 10^5 \times [\text{cytochrome } c]\ (M) + 0.67$$

and

$$E_p(A_{II})\ (V) = 5.26 \times 10^5 \times [\text{cytochrome } c]\ (M) + 0.87.$$

After reaching the maximum value of the anodic peaks, further increase in cytochrome *c* concentration had no influence on the potential of these peaks.

The dependence of the height of peaks A_I and A_{II} on E_i was also recorded (fig. 4). Whereas the height of peak A_I was independent of E_i in the whole range of E_i used (0.2– −0.6 V), the height of the more positive peak A_{II} was independent of E_i only at E_i ranging from 0.2 to approx. −0.1 V. If the value of E_i was further changed to more negative values, peak A_{II} was lowered; at $E_i = -0.6$ V it practically disappeared. The potentials of peaks A_I and A_{II} were, however, independent of E_i.

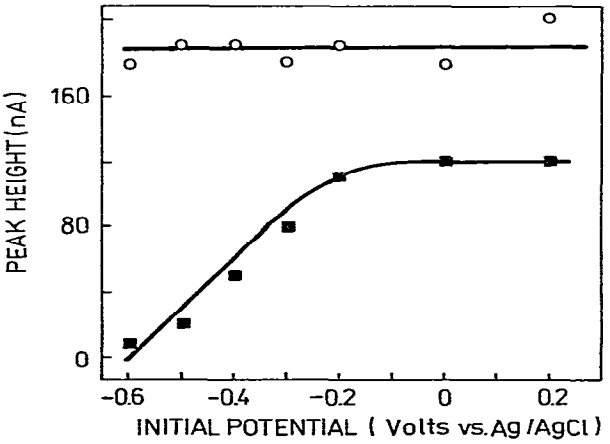

Fig. 4. Variation of the height of DP voltammetric peaks A_I (○) and A_{II} (■) of 1.6×10^{-7} M cytochrome *c* with the initial potential at the WISGE. Other conditions were the same as in fig. 1b.

3.2. Cyclic voltammetry

CV of 2.1×10^{-8} M ferricytochrome *c* at the WISGE was carried out in two ways, designated CV-I and CV-II: If CV-I was employed, an E_i of 0.2 V was used and the first, forward potential sweep was cathodic; after the potential had reached a value of −0.8 V (switching potential), the voltage sweep was immediately reversed so that E_i could be reached. If CV-II was employed, an E_i of 0.0 V was used and the first, forward potential sweep was anodic; switching potential was 1.1 V.

CV-I forward (cathodic) sweep gave a single, well developed peak at potentials around −0.4 V (fig. 5a). The height of this peak was independent of pre-sweep (waiting) time at $E_i = 0.2$ V in the range 10–360 s. In the course of the reverse part of the current-potential curve, this peak had no anodic counterpart in the whole region of potentials used in CV-I experiments. The height of the cathodic peak increased with growing voltage scan rate (v) (fig. 6a). At low scan rates (up to approx. 0.010 V s^{-1}) the height of the peak was directly proportional to v. At higher scan rates the dependence of cathodic peak height on v tended to a limiting value. The peak potential was shifted with increasing v to more negative values (fig. 6a).

Repetitive cyclic scans on the same graphite electrode surface at a constant scan rate (0.050 V s^{-1}) yielded a shift of the cathodic peak towards more positive potentials and a decrease in the peak current (fig. 5 and table 2). This decrease was more marked with lower scan rate (table 2). The repetitive cyclic scans were also performed in such a way that, after recording the first cathodic part of a CV-I curve, the WISGE was held for various times at −0.8 V, and only after this waiting time were the anodic part of the first cycle and the second cathodic potential sweep recorded. The increase in this waiting time at −0.8 V led to very effective lowering of the cathodic CV peak height at −0.4 V on the voltammetric curve corresponding to this second potential sweep (table 2).

In further studies, the method for the investiga-

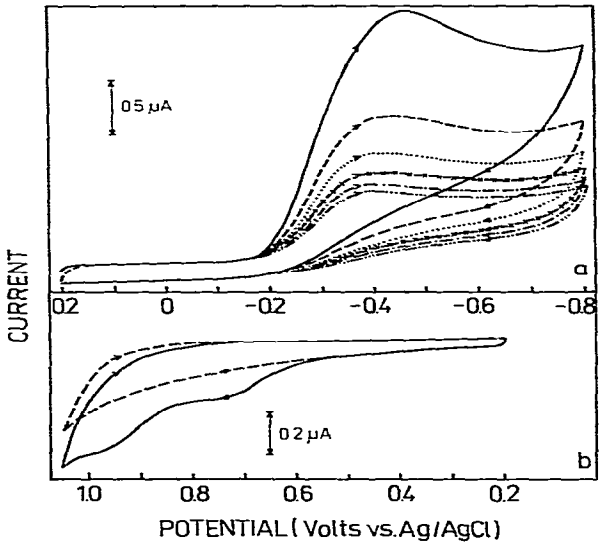

Fig. 5. Influence of repetitive cyclic scans on the same WISGE surface. Cyclic voltammetry of cytochrome *c* at a concentration of 2.1×10^{-8} M in 0.1 M potassium phosphate, pH 7.0, was performed at a voltage scan rate of 0.050 V s^{-1}. (a) Initial potential was 0.2 V; first,forward potential sweep was cathodic. (b) Initial potential was 0.0 V; first, forward potential sweep was anodic. Waiting time at the initial potential was always 120 s. (———) First scan, (- - - - -) second scan. (· · · · · ·) third scan, (×———×) fourth scan, (· — · — ·) fifth scan, (– · · · –) sixth scan.

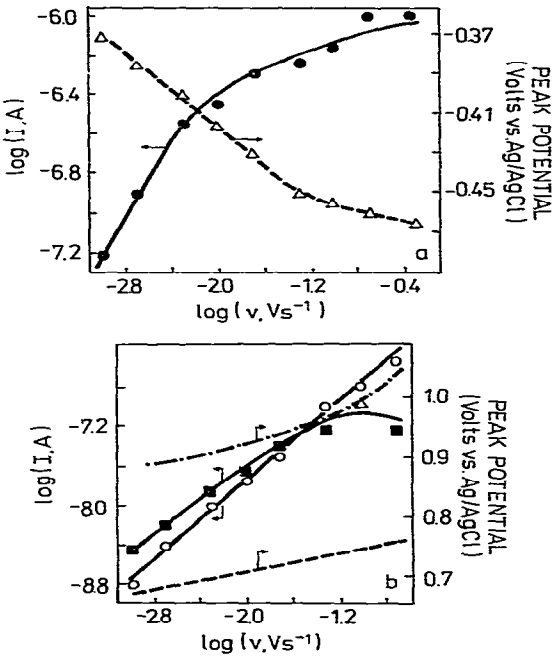

Fig. 6. Effect of voltage scan rate, *v*, on the CV behaviour of 2.1×10^{-8} M cytochrome *c* at the WISGE. (a) Cathodic peak height (●), cathodic peak potential (△); other conditions were the same as in fig. 5a. (b) More negative anodic peak height (○), more positive anodic peak height (■), more negative anodic peak potential (- - - - -), more positive anodic peak potential (· — · — ·). Other conditions were the same as in fig. 5b.

Table 2

The influence of repetitive cyclic scans on the behaviour of cytochrome *c* at the WISGE at various voltage scan rates (*v*)

Voltage scan rate is expressed as V s^{-1}.

	Cathodic peak height (%) [a]				
	v = 0.200	*v* = 0.050			*v* = 0.002
1st potential sweep	100	100	100	100	100
2nd potential sweep	61	56	14 [b]	9 [c]	53
3rd potential sweep	33	27			25

[a] Ferricytochrome *c* at a concentration of 2.1×10^{-8} M in 0.1 M potassium phosphate, pH 7.0. Initial potential of 0.2 V, first, forward potential sweep was cathodic, switching potential was −0.8 V.

[b] The waiting time at −0.8 V (after recording the cathodic part of the first potential cycle) was 5 min.

[c] The waiting time at −0.8 V (after recording the cathodic part of the first potential cycle) was 20 min.

tion of cytochrome *c* adsorption at the WISGE was used, which is sometimes designated the 'film transfer method' [4,10]. The experiments performed with the aid of this technique were carried out in deoxygenated solutions containing 2.1×10^{-8} M ferricytochrome *c* in 0.1 M potassium phosphate, pH 7.0. At this concentration 120 s were allowed for the adsorption. If thus-equilibrated WISGE was washed and transferred to a deoxygenated solution of 0.1 M potassium phosphate, pH 7.0, free of cytochrome *c*, its CV-I behaviour was identical to that observed in the solution of 2.1×10^{-8} M ferricytochrome *c*. Repetitive cyclic scans on the same graphite electrode surface coated with ferricytochrome *c* yielded the same results as the same experiment carried out in the presence of 2.1×10^{-8} M ferricytochrome *c* (fig. 5a and table 2). If the coated electrode was taken out of the cell (e.g., after the sixth cycle), exposed for 60 s in air, then transferred again into deoxygenated solution free of cytochrome *c*, after new shorter deoxygenation the cathodic peak was obtained, the height of which was approximately identical to that obtained on the first cyclic voltammogram (using the first cathodic potential sweep).

CV-I behaviour of ferricytochrome *c* at the WISGE was also observed in the presence of 4,4'-bipyridyl in a medium of 0.1 M potassium phosphate, pH 7.0. The concentration of 4,4'-bipyridyl (0.01 M) was identical to that used by Eddowes et al. [3] in their experiments with a gold electrode. No peaks appeared on cyclic voltammmograms in the region of potentials from 0.4 to −0.3 V.

CV-II forward (anodic) potential sweep gave two peaks (fig. 5b) at potentials around 0.70 and 0.95 V. The height of these peaks was independent of pre-sweep time at an E_i of 0.0 V in the range 10–360 s. In the course of the reverse part of the current-potential curve these peaks had no cathodic counterparts (fig. 5b). The heights of both these anodic peaks increased linearly with growing voltage scan rate. The dependence of the more positive peak height on scan rate at higher scan rates (above 0.020 V s^{-1}) started to tend towards a limiting value (fig. 6b). The peak potential of both anodic CV peaks was shifted with growing scan rates to more positive values (fig. 6b). Repetitive cyclic scans on the same graphite electrode surface yielded no peak on current-potential curves (fig. 5b).

The 'transfer film method' was used also in CV-II experiments. Other conditions of this CV-II measurements were identical to those used in transfer film experiments performed with the aid of CV-I. After the equilibration of the WISGE and its washing in cytochrome *c*-free solution, the CV-II behaviour of the coated electrode was identical to that observed in the solution containing ferricytochrome *c*.

4. Discussion

4.1. The nature of cathodic reactions of cytochrome c at graphite electrodes

The results of the concentration dependences (fig. 3) and the film transfer method indicate that cytochrome *c* is adsorbed at the graphite electrode and that its electrochemical activity described in this report is limited only to the layer of the adsorbed protein next to the electrode.

It follows from the courses of DP voltammograms of all proteins investigated in this work that an irreversible electrochemical reaction is responsible for the appearance of the cathodic voltammetric peak C (figs. 1a and 5a) in which a key role is played by haemin iron bound in the protein. The potential of this peak is not very different from that of one-electron reduction of cytochrome *c* haemin iron at a mercury electrode [10,21]. If the latter reaction were responsible for the appearance of the cathodic peak C, the area under the CV-I cathodic peak should correspond to approx. 9.4×10^{-8} C*. The area under the cathodic CV curve (the first trace on fig. 5a) corresponds, however, to

* The calculation for this quantity of charge transferred was performed as follows: number of coulombs transferred = $PnFA$, where P is the number of moles of cytochrome *c* present in 1 cm^2 of the adsorbed monolayer at full coverage of the electrode having area A; n is the total number of electrons involved in electrode reaction and F equals 96500 C; P was estimated on the basis of a value of 12.0 nm^2 for the area occupied by one ferricytochrome *c* molecule at the electrode surface [22].

about 8.3×10^{-6} C, i.e., a value roughly 90-times higher than that expected. It is, therefore, possible to assume that peak C corresponds to a catalytic reaction. Haem is known to catalyze reduction of oxygen at various electrodes [23–26]. Even though electrochemical measurements in this work were performed in carefully deoxygenated solutions, catalytic reduction of oxygen adsorbed at the graphite surface could take place. It is known [27,28] that oxygen is adsorbed on the surface of graphite and that it is difficult to remove it from the surface of graphite [27]. For instance, bubbling the solution into which the graphite is immersed with inert gas is not effective in this respect. However, even this possibility of catalytic electroreduction of oxygen does not seem to be very likely. As described in the report of Kolpin and Swofford [23] this catalytic activity is performed by haem (having iron in the bivalent reduced form) and not by haemin. The chemically reduced cytochrome *c* yields, however, a markedly lower peak C than ferricytochrome *c* under identical experimental conditions. Further, pyrolytic graphite is known to have very low porosity in comparison with spectroscopic graphite [27]. It is, therefore, possible to expect that also the amount of oxygen adsorbed at the surface of the PGE is markedly lower than at the surface of the WISGE. Thus, it follows from comparison of the heights of DP voltammetric peak C obtained with the aid of the WISGE and PGE (see section 3.1.) that oxygen adsorbed at graphite plays a certain role in the electrochemical reaction responsible for the origin of peak C.

It seems, therefore, reasonable to suggest that a higher current of ferricytochrome *c* at the WISGE (as compared with that expected for simple one-electron reduction of ferricytochrome *c* to ferrocytochrome *c*) could be caused by regeneration of ferricytochrome *c*, with oxygen adsorbed at the WISGE surface. In this way, a given ferricytochrome *c* molecule may be reduced at the WISGE several times during one cathodic potential sweep. The increase in current thus occurs at the expense of the adsorbed oxygen, which on its own is chemically a more powerful oxidizing agent than ferricytochrome *c*. The current increase would be thus related to the rate of the reaction between adsorbed oxygen and ferrocytochrome *c*.

As follows from the crystallographically estimated tertiary structure of cytochrome *c* [1], the prosthetic group is hidden inside the protein molecule. It appears, therefore, likely that the suggested catalytic reaction takes place only after haemin becomes accessible for reaction with the electrode. Haemin bound in the cytochrome *c* molecule could become accessible due to unfolding or flattening of the protein molecule adsorbed at the WISGE charged to the potentials of peak C. Such a process has already been described also for other globular proteins at mercury/solution or air/solution interfaces [29]. A particular role in these interfacial conformational changes could be played by the electric field in the close proximity of the WISGE charged to negative potentials. Further, the course of the dependence of the height of the CV-I cathodic peak on scan rate (fig. 6a) is apparently connected with the fact that the process including the interfacial conformational change and the reaction between adsorbed ferrocytochrome *c* and oxygen is a relatively slow process. It is, therefore, possible that during the first cathodic polarization sweep this surface conformational change does not take place in all cytochrome *c* molecules adsorbed at the WISGE. The appearance of peak C on curves corresponding to repetitive cyclic scans could be thus connected not only with reoxidation of some cytochrome *c* molecules by adsorbed oxygen during the reverse part of the foregoing cycle, but also by the conformational change leading to the accessibility of haemin for the reaction with the electrode in further adsorbed cytochrome *c* molecules.

4.2. The nature of anodic reactions of cytochrome c at graphite electrodes

Non-conjugated proteins studied in this work yield an anodic DP voltammetric peak at a potential of 0.66 V (fig. 2). This peak corresponds to the electrochemical oxidation of tyrosine residues [11,12]. Cytochrome *c*, the molecule of which contains four tyrosine residues [1], yields peak A_I at the same potential (fig. 2). It seems, therefore, reasonable to ascribe this peak of cytochrome *c* also to electrooxidation of its tyrosine residues. Ferricytochrome *c* yields, however, the more positive

peak A_{II}, which appears at potentials markedly more positive than the second, more positive DP voltammetric peak of non-conjugated proteins. It has been shown [11,12] that this second peak of non-conjugated proteins corresponds to electrochemical oxidation of their tryptophan residues. Since peak A_{II} is yielded only by haemin-containing proteins, its origin can be attributed to the electrode reaction in which haemin or haem participates. Work aimed at understanding this reaction is in progress and will be published elsewhere.

The dependence of anodic peak A_{II} of ferricytochrome *c* on the initial potential reveals that the height of this peak starts to decrease if the electrode is pre-polarized for 120 s to potentials corresponding to cathodic peak C (fig. 4). This result could be connected with the fact that reduction of ferricytochrome *c* at the WISGE leads to a change of the conformation of this adsorbed protein. The consequence of this conformational change could be a change in the configuration of haem on the electrode surface that would not be favourable to the electrode reaction responsible for the appearance of peak A_{II}. The independence of the height of ferricytochrome *c* peak A_I on the initial potential in the range -0.6 to 0.2 V could indicate that this conformational change does not influence the accessibility of tyrosine residues for reaction at the electrode in the adsorbed cytochrome *c* molecule.

In conclusion, it is possible to summarize that this report demonstrates the applicability of graphite electrodes to electrochemical analysis of conjugated proteins containing electrochemically active groups. The use of graphite electrodes for this purpose makes it possible to investigate particularly anodic reactions of these proteins which cannot be studied, for instance, with the aid of the mercury electrode used in polarographic analysis. Nevertheless, this report demonstrates also that the application of graphite electrodes even to investigation of cathodic processes can provide new information about the interactions of these biologically important macromolecules with electrically charged surfaces.

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