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SHORT COMMUNICATION

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Direct electrochemistry of bovine heart cytochrome *c* facilitated by cysteine derivatives and analogues. Some effects of facilitator structure

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Abstract Bovine heart cytochrome *c* electrochemistry was investigated by cyclic voltammetry using gold electrodes modified by self-assembled monolayers of cysteine and some of its derivatives, including glutathione. Glutathione shows a peculiar dependence of the facilitating ability on the oxidation state of the sulfur functionality. Whereas the disulfide form acts as an efficient facilitator, the thiol form is completely inactive. This behavior was accounted for by the lower stability of adsorbed thiol layers as compared to disulfide layers. Apparently, small molecules of cysteine derivative form rather stable adsorbed layers with a high degree of dimer formation. Conversely, reduced glutathione is not able to turn into the disulfide form in the adsorbed layer. Consequently, only the layer produced by the adsorption of the disulfide form itself is stable enough for promoting direct electron transfer reactions of cyt *c*.

Keywords Cytochrome *c* · Electrochemistry · Cysteine · Glutathione · Modified electrode

Introduction

Cytochrome *c* (cyt *c*) is a small heme protein involved in electron transport within the living cell [1]. This function stimulated the investigations on the electrochemistry of cyt *c* as a prototype of redox proteins. Unfortunately, electron transfer (ET) between a bare metal electrode and cyt *c* molecules is hindered by protein adsorption leading to the self-blocking of the electrode surface, as well as by the lack of appropriate molecule orientation at the bare metal electrode surface [2]. In order to investigate the direct ET, functionalized electrodes that display negative functionalities should be used. Electrostatic interactions between the modifier layer and the positively-charged protein domain stimulate the favorable orientation at the electrode surface and also prevent the irreversible, random adsorption of the protein itself [3]. This finding has considerable consequences for the development of the experimental methods in the field of redox protein chemistry [4] and also opens a promising route to the application of redox proteins as mediators in amperometric enzyme sensors [5].

A straightforward method for electrode functionalization consists in the spontaneous formation of a self-assembled thiol layer on a gold surface [6]. This method was largely employed to facilitate the direct ET between redox proteins and metal electrodes [3, 7, 8]. Although quite complicated molecules were initially used as electrode modifiers, it was shown subsequently that a self-assembled monolayer (SAM) of long-chain mercaptocarboxylic acid is also an efficient facilitator. Such a facilitator enables the investigations of cyt *c* molecules confined to the electrode surface by either irreversible adsorption [9] or covalent binding to the modifier layer [11], with the particular advantages provided by the diffusionless electrode process.

One of the reasons for investigating cyt *c* electrochemistry on modified electrodes is the assumption that, in terms of protein-protein interaction, the modifier layer mimics the behavior of the biological ET partner of cyt *c*. However, a facilitator layer with a uniform composition (e.g., an adsorbed thiocarboxylic acid) could replicate the characteristics of the partner to a limited extent only, depending on structure features of the protein [10]. Therefore, one concluded that SAMs of constant chain length and

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low defect density are unlikely to be the surface of choice for binding ET proteins with optimal electronic coupling [10]. Conversely, multifunctional facilitators may provide a more realistic simulation of the behavior of binding sites in the cyt *c* protein partner. That is why sulfur-containing amino acids [12, 13] and peptides [14] play a special role as facilitators in cyt *c* electrochemistry.

This paper presents an investigation of cysteine derivatives as ET facilitators in the light of the recent progress in the field of thiol SAMs. A special emphasis is put on the facilitator behavior of the wide-spread peptide glutathione.

Materials and methods

Bovine heart cyt *c* (Sigma, 95%) was purified by chromatography at 4 °C over a cation exchange resin (Sephadex CM-25, 14 × 2 cm column). The compounds used as modifiers (Table 1) were Sigma products, except for SSbipy (Aldrich). Polycrystalline gold wire (Aldrich, 99.999%) was sealed with epoxy resin into a glass tube leaving a disk of 0.5 mm diameter as the active area. The electrode surface was prepared according to well-known procedures [15, 16]. *Ex situ* modification of the surface was performed by dipping the electrode for 30 min. in the aqueous solution of the facilitator (either 1 mM or at saturation if the facilitator is sparingly soluble). The supporting electrolyte in the voltammetric experiments consisted of 26 mM Na_2HPO_4, 20 mM NaH_2PO_4, and 0.1 M $NaClO_4$ (pH 7). Voltammetric curves were recorded by means of PAR 264A equipment. All potential values are reported with respect to the SCE. More experimental details are provided elsewhere [15]. The heterogeneous rate constants were determined by the Nicholson method [17].

Results and discussion

Table 1 summarizes the parameters of cyclic voltammograms recorded for cyt *c* ET reaction at the gold electrode modified with various compounds, mostly cysteine derivatives. In some instances, the voltammetric response is not peak-shaped but shows a sigmoid pattern. That is why characteristic potentials are reported as half peak potentials (i.e., the potential for the $i_p/2$ current, where i_p is the peak current). The most probable orientation of the modifier molecule at the gold electrode surface is displayed in Fig. 1.

Typical voltammograms for bovine cyt *c* recorded with various modifiers are shown in Fig. 2. MP, which is one of the first facilitators employed as an irreversibly adsorbed layer in direct electrochemistry of cyt *c* [16, 18], shows the expected behavior (Fig. 2, A and Table 1), with a standard rate constant of $3.8 \cdot 10^{-3}$ cm·s^{-1}, in a good agreement with published data [19]. However, the additional riboside group in MPR completely removes the promoting capacity and only the background current is recorded in the presence of this compound (Fig. 2, A). This effect is opposite to that expected if only the negative charge of the polar hydroxy groups is taken into account. In other words, the sugar fragment "hides" the rest of the molecule. Such an effect points out the importance of the steric factors in addition to the polarity of the facilitator molecule.

Among the cysteine derivatives, glutathione (γ-L-glutamyl-L-cysteinyl-glycine) showed the most intriguing behavior. The reduced form (GSH) is not able to facilitate the cyt *c* redox reaction (Fig. 2, B), although it can form a monolayer assembly on the gold electrode [20]. Conversely, the disulfide form (GSSG) exhibits good facilitator properties (Fig. 2, B), mostly similar to those of SSbipy which is a typical facilitator [21]. The ET reactions of cyt *c* on the GSSG-modified electrode are diffusion controlled, as proved by the effect of the scan speed (v). Thus, both cathodic and anodic peak currents are directly proportional to $v^{1/2}$ with slopes of about 0.01 μA·mV$^{-1/2}$ s$^{1/2}$ (for v ranging between 5 and 100 mV·s^{-1}). This demonstrates the reversible character of cyt *c* binding to the GSSG-modified electrode surface.

The first scan results in peak-shaped patterns (Fig. 2, B) but after subsequent cycling, the shape of the voltammogram turns to a sigmoid one (Fig. 3, curves 1 to 3). Such an effect can be accounted for by a change in the state of the GSSG-modified electrode surface. It may turn from a homogeneous structure to a collection of insular active regions that behaves like a microelectrode array. Under these circumstances, radial diffusion occurs instead of the linear one and the shape of the voltammogram shifts to the typical form for the steady state

Table 1 Characteristics of the cyclic voltammetric patterns for cyt *c* using various facilitators[a]

Facilitator/Modifier	$E_{c,1/2}$ (mV)	$E_{a,1/2}$ (mV)	E_0 (mV)	i_c/i_a	Stability at successive cycling
4,4′-Dipyridyl disulfide (SSbipy)	18.3	20.5	19.4	1.11	Good
6 Mercaptopurine (MP)	22.7	22.7	22.7	1.03	Good
6 Mercaptopurine riboside (MPR)	–	–	–	–	No signal
Glutathione disulfide (GSSG)	18.3	20.5	19.4	0.94	Good
Glutathione (GSH)	–	–	–	–	No signal
Penicillamine (Pen)	22.7	22.7	22.7	1.10	Good
Cysteine (Cys)	38.3	13.8	26.1	1.22	Poor
Cystine [$(Cys)_2$]	33.8	36.1	34.9	2.17	Poor
Homocysteine (Hcys)	–80.5	22.7	–28.9	2.79	Poor
Cysteinyl-glycine (Cys-Gly)	27.2	33.8	30.5	1.04	Poor

[a] $E_{c,1/2}$, $E_{a,1/2}$ = half-peak potentials for the cathodic and anodic peak, respectively
$E_0 = (E_{c,1/2} + E_{a,1/2})/2$ is a rough estimation of the formal redox potential

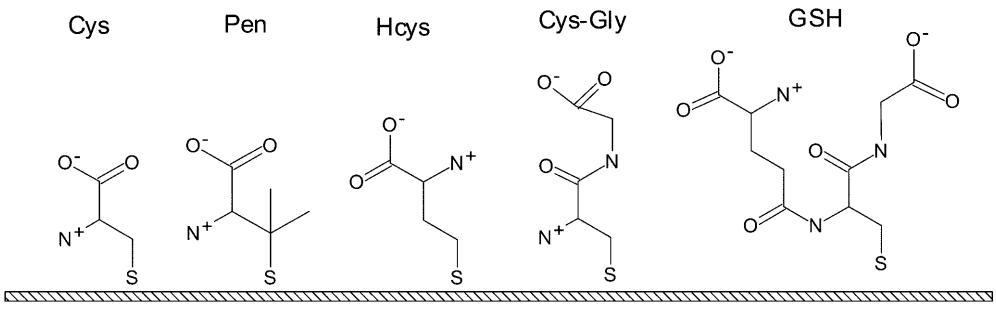

Fig. 1 Schematic representation of cysteine-like modifiers at the gold electrode surface

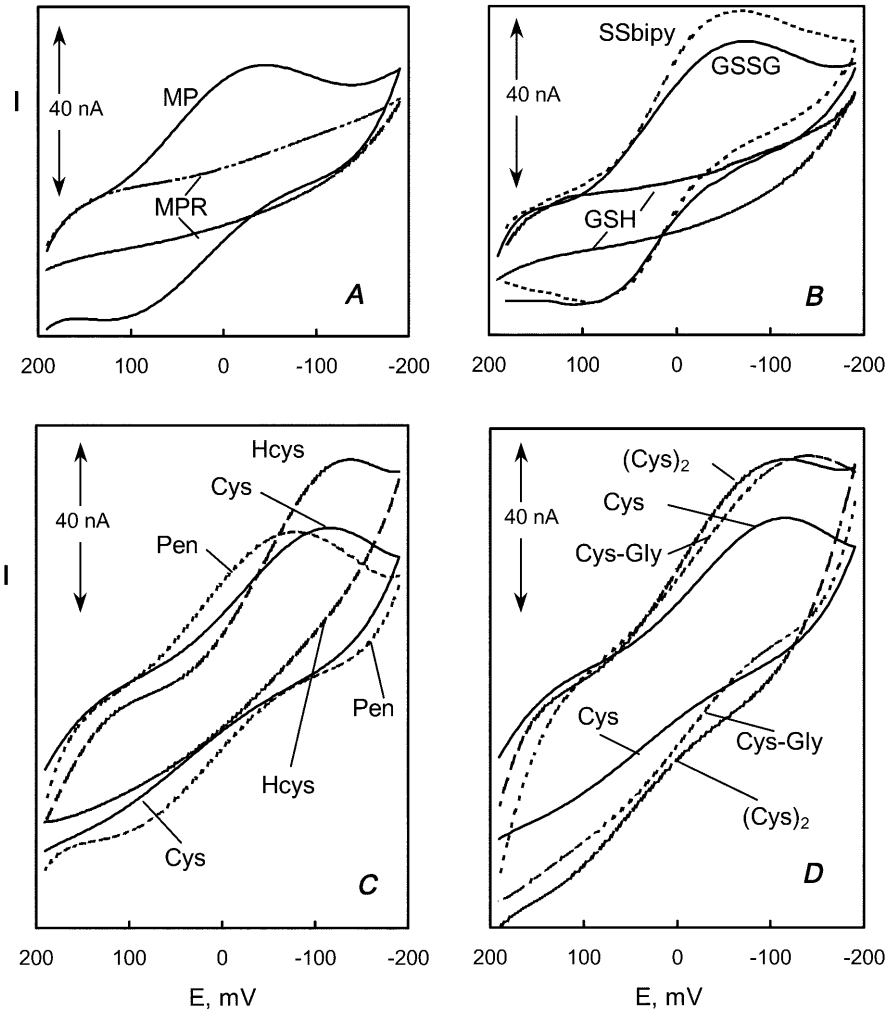

Fig. 2A–D Voltammetric signals corresponding to bovine cyt *c* (0.56 mM) in phosphate buffer (pH 7) on the gold electrode modified with various sulfur organic compounds. Scan speed: 20 mV s^{-1}

mass transfer (Fig. 3, curves 2 and 3). This interpretation is in accord with the microscopic model advanced by Armstrong, Bond and Hill [7, 22, 23, 24]. As pointed out by Bond, "…if significant change in wave shape is observed at the reversible potential it is most likely that it is the nature of the electrode surface and therefore the mass transport process that is being altered and not the rate of electron transfer" [23]. However, an increase in irreversibility cannot be completely ruled out. The sigmoid form is rather stable, as the shape of the voltammogram remain unchanged during the 50 subsequent cyclic potential scans. However, after a prolonged contact (of about 15 h) of the modified electrode with the cyt *c* solution, the facilitator is substituted by adsorbed protein molecules and the cyt *c* voltammetric signal becomes very meager (Fig. 3, curve 4).

Under the conditions of the present work, the heterogeneous rate constant is $8{\cdot}10^{-4}$ cm s^{-1} for GSSG and $4.5{\cdot}10^{-3}$ cm s^{-1} for SSbipy. The last figure compares favorably with literature data ($5{\cdot}10^{-3}$ cm s^{-1}), [25]), whereas the value for the GSSG is quite close to the constants reported for L-cystinyl-bis(L-glycine) ($1{\cdot}10^{-3}$ cm s^{-1}) and L-cystinyl-bis(L-glutamic acid), [$(Cys\text{-}Glu)_2$, $1.5{\cdot}10^{-3}$ cm s^{-1}] [14].

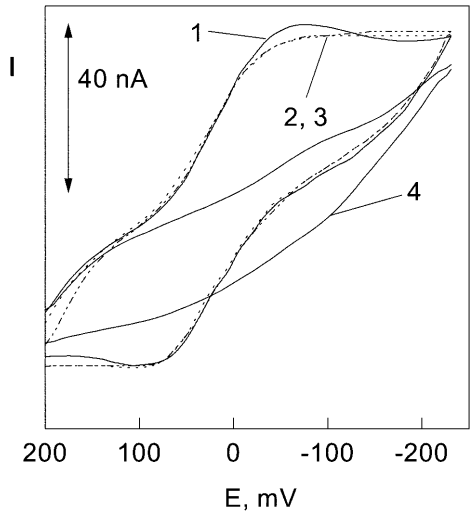

Fig. 3 Effect of successive scanning on the cyt *c* voltammetric response on the GSSG modified gold electrode. Same conditions as in Fig. 2. Curves 1–3: first 3 scans. Curve 4: CV scan after holding the electrode for 15 hours in contact with the cyt *c* solution at open circuit

The facilitator activity of GSSG, as described in this work, is mostly similar to that of $(Cys\text{-}Glu)_2$ and Cys-Gly [14], except for the lower stability of the adsorbed Cys-Gly layer (Table 1). However, Cys-Gly is a good facilitator, not only in the thiol (Fig. 2, C and Table 1) but also in the disulfide form [14]. Likewise, the facilitator activity of Cys and CySSCy are somewhat similar (Fig. 2, D, Table 1), i.e., not essentially dependent on the oxidation state of the sulfur atoms in the dissolved state. The absence of some noticeable differences in the cases of the Cys/$(Cys)_2$ and Cys-Gly/$(Cys\text{-}Gly)_2$ strongly contrasts the behavior of the GSH/GSSG couple as described before.

Table 1 also shows some differences in the stability of the voltammetric response at successive cycling as a function of facilitator structure. The stability of the response depends on the stability of the modifier layer towards the substitution by protein molecules adsorbed in an electrochemically inactive orientation. The same effect may decide about the value of the cathodic to anodic peak current ratio. A ratio value close to 1 demonstrates a very slow substitution of the modifier whereas a higher value proves significant deactivation at the time scale of the experiment. From this standpoint, GSSG behaves mostly like some standard modifiers, such as MP and SSbipy. Conversely, the ET reaction facilitated by smaller molecules like Cys, $(Cys)_2$, and Cys-Gly is far from reversibility, as demonstrated by the i_c/i_a ratio and/or by the shift of the formal redox potential from the expected value of +0.18 V vs. SCE [26]. From this standpoint, Pen forms an exception, probably because the hydrophobic methylene groups increase the stability of the surface facilitator layer by preventing facilitator substitution by protein molecules adsorbed in a non-favorable orientation (Fig. 1). Also, hydrophobicity might just as well add to protein adsorption on the Pen-modified electrode surface. On the other hand, the longer and more flexible molecule of Hcys gives a less stable SAM and the relevant voltammogram is more flattened (Fig. 2, C).

As the protein-modifier interaction is electrostatic in nature, the overall charge of the modifier molecule is an important parameter. In order to assess its effect, it may be assumed that the sulfur atom, which is involved in a quasi-covalent bond with the gold surface [6], does not contribute to the overall molecule charge. In this instance, the overall charge of thioamino acids and Cys-Gly is null at pH about 7, where both amino and carboxyl groups are ionized. Although the zwitterionic form enables the mutual compensation of the electrical charges the molecule still preserves a strong polar character and keeps the negative end oriented towards the solution (Fig. 1). On the other hand, the occurrence of ionic groups in the facilitator molecule could enable the insertion of water dipoles into the facilitator layer thus decreasing the density of adsorbed molecules. This makes more probable the substitution of the modifier by adsorbed protein molecules leading to the gradual deactivation of the electrode surface. In this connection, the better modifier behavior of Pen (Table 1) could be assigned to the hydrophobicity of the methyl groups, in addition to the pure steric hindering. Attempts at determining the amount of adsorbed facilitator by extending the cathodic range in the CV run were ineffective. Apparently, the cathodic desorption occurs beyond the limit of hydrogen ion reduction in the phosphate buffer.

It is also interesting to note the difference in the formal potential when comparing GSSG on one hand and other thiolic facilitators in Table 1, on the other hand. The lower value detected in the presence of GSSG may be due to the stabilization of the oxidized form in contact with the negatively charged electrode surface. Such an effect is analogous to that produced by anions in solution [27].

In contrast with the above compounds, the GSH molecule, which displays an overall negative charge, does not facilitate the cyt *c* ET reaction at all. An explanation of this behavior can be attempted by taking into account the nature of the sulfur-gold interaction in the SAM. In this respect, it is important to point out the results of an X-ray diffraction study demonstrating that the sulfur end groups of adsorbed alkanethiol assume a dimerized disposition on the surface of Au(111) [28]. Furthermore, disulfide formation in an alkanethiol SAM was directly proved by thermal desorption mass spectroscopy [29, 30]. This does not exclude the occurrence of plain thiols as adsorbed species when the coverage degree is very low [30]. The main expected effect of the difference in the state of the sulfur functionality is a higher stability of the adsorbed disulfide form due to the distribution of the electron density among 4 centers (-Au-S-S-Au-) instead of only two (-Au-S-) in the case of an adsorbed thiol [31].

Table 2 Effect of modification and measurement temperatures on cyt *c* peak currents recorded on electrodes modified by either SSbipy or GSSG. Currents values are relative to those obtained when both the modification and the voltammetric run occurred at 24 °C

Modification/recording temperature, °C	SSbipy $i_c i_a$		GSSG $i_c i_a$	
24/24	1	1	1	1
24/4	0.75	0.72	0.73	0.79
4/24	0.87	0.81	1.41	1.47
4/4	0.82	0.81	0.80	0.84

The above findings refer to simple alkanethiols but can be readily extrapolated to Cys and its derivatives if the effects of the additional structure details are taken into account. It appears that, except for GSH, all the investigated Cys derivatives (Table 1) may turn into the disulfide form after adsorption, giving the resulting SAM enough stability, at least for the time interval of a double scan run. Conversely, steric factors as well as electrostatic repulsion prevent GSH molecules from approaching each other at the distance required for the formation of the -S-S- bond and a plain thiol layer results. Such a layer is stable enough to hinder small inorganic species from approaching the electrode [20] but is quickly damaged by protein adsorption. Conversely, GSSG seems to preserve in the adsorbed form the preexisting disulfide state. The resulting SAM is much more stable than that produced by GSH adsorption and facilitates cyt *c* ET.

A test for the above interpretation is provided by the effect of the temperature during the electrode modification and the voltammetric run, respectively (Table 2), using either GSSG or SSbipy, the latter as a reference modifier [21]. Peak currents values in this Table are relative to those obtained when both modification and the voltammetric run occurred at 24 °C. If the current is recorded at 4 °C, peak current values are lower than under the reference conditions, probably due to the slower orientation of the cyt *c* molecule into the favorable position for the occurrence of ET. If the voltammograms are recorded at 24 °C after modification at 4 °C, GSSG shows an increased activity that can be accounted for by the favorable effect of the lower temperature on the adsorbed layer density and orientation. SSbipy shows an opposite behavior as a possible consequence of a higher activation energy for the formation of the sulfur-gold bond. Finally, if both steps occur at 4 °C, both modifiers show a slightly depressed activity, proving the preponderance of the kinetic factors in the ET process over the effect of some structural details of the modifier layer.

Conclusions

Summing up, it is found that the facilitator activity in cyt *c* electrochemical reactions can be rationalized by taking into account not only the overall electrical charge but also the ability of the thiolic facilitator to turn into the disulfide state by adsorption on the gold electrode surface. Steric factors as well as electrostatic interactions could prevent the arrangement of the thiolic facilitator in an adsorbed disulfide form. In this respect, cyt *c* electrochemistry can be a probe for the oxidation state of sulfur functionality in the SAM of a thiol containing additional ionic or polar groups.

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