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Direct electron transfer reactions between human ceruloplasmin and electrodes

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ABSTRACT

In an effort to find conditions favouring bioelectrocatalytic reduction of oxygen by surface-immobilised human ceruloplasmin (Cp), direct electron transfer (DET) reactions between Cp and an extended range of surfaces were considered. Exploiting advances in surface nanotechnology, bare and carbon-nanotube-modified spectrographic graphite electrodes as well as bare, thiol- and gold-nanoparticle-modified gold electrodes were considered, and ellipsometry provided clues as to the amount and form of adsorbed Cp. DET was studied under different conditions by cyclic voltammetry and chronoamperometry. Two Faradaic processes with midpoint potentials of about 400 mV and 700 mV vs. NHE, corresponding to the redox transformation of copper sites of Cp, were clearly observed. In spite of the significant amount of Cp adsorbed on the electrode surfaces, as well as the quite fast DET reactions between the redox enzyme and electrodes, bioelectrocatalytic reduction of oxygen by immobilised Cp was never registered. The bioelectrocatalytic inertness of this complex multi-functional redox enzyme interacting with a variety of surfaces might be associated with a very complex mechanism of intramolecular electron transfer involving a kinetic trapping behaviour.

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1. Introduction

Ceruloplasmin (ferroxidase, iron(II):oxygen oxidoreductase, EC 1.16.3.1) is one of the most complex blue multicopper oxidases [1,2]. The complete function of human ceruloplasmin (Cp) is not fully understood because of its sheer complexity – it shares functional characteristics with ferroxidase, amine and NO oxidase, and nitrite synthase, displays antioxidant activity, and is associated with copper and iron transport in the human body [3–8]. Knowledge on the general redox properties of Cp might give insights into the possible roles of this multi-functional enzyme in the human body [3–5,8], which is an important question because of the medical significance of the protein first suggested in 1952 [9]. Moreover, electrochemical investigations of direct electron transfer (DET) reactions of Cp might enable the development of biocompatible and efficient cathodes for potentially implantable biofuel cells [10,11], if bioelectrocatalytic reduction of O₂ by the protein is achieved.

Human Cp is a 132 kDa monomer with a significant amount of glycosylation (7–8%) [12]. In contrast to the three-domain structures

of ascorbate oxidase (AOx), bilirubin oxidase (BOx), and laccase (Lc) containing four copper ions, Cp has six copper ions and is comprised of six compact β -barrel domains, with large loop insertions, giving the protein a unique triangular symmetry (Fig. 1).

However, the configuration of the copper ions is similar to that of other blue multicopper oxidases. In Cp, three copper ions are situated in the T2/T3 cluster and three ions are bound to the three T1-binding sites [12]. The three copper ions of the trinuclear cluster lie at the interface between the first and last domains, 1 and 6 respectively, possessing ligands from each domain, an arrangement also seen in the structures of AOx [13] and Lc [14] (Fig. 1). The remaining three copper ions are mononuclear centres called T1_{Remote}, T1_{CysHis}, and T1_{PR}, held by intra-domain sites. T1_{Remote} and T1_{CysHis}, located in domains 4 and 6 respectively, have a typical T1 copper environment with a set of four ligands: two histidines, one methionine and one cysteine (Fig. 1). The T1_{PR} site, located in domain 2, has a different ligand structure in that it lacks the methionine, which is replaced in the amino acid sequence by a leucine residue, Leu329, analogous to the T1 site of some fungal Lcs [15]. The T1_{CysHis} centre is connected to the T2/T3 cluster via the highly conserved Cys-2His electron transfer (ET) pathway, also found in the structures of AOx [13] and Lc [14], across a distance of approximately 13 Å (Fig. 1A). It is widely held that O₂ is bound and reduced to two H₂O molecules at the three-nuclear copper cluster, whereas the mononuclear T1_{HisCys} site is able to accept electrons from reduced substrates of Cp, e.g. Fe(II) (Fig. 1A) [2,15]. In spite of the fast

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Fig. 1. (A) Proposed mechanisms of Fe(II) oxidation and O₂ reduction by human Cp. The three-dimensional structure of the enzyme was visualised using the program PyMOL v. 0.99 (PDB 1KCW). The six domains are colour coded as red (1), blue (2), green (3), light blue (4), yellow (5), and purple (6). Six copper ions are shown in cyan and carbohydrates (*n*-acetyl-*d*-glucosamine) are in black. (B) Schematic view of a surface-adsorbed Cp molecule consistent with a roughly native conformation (i.e. not significantly denatured).

electronic communication between T1_{HisCys} and T1_{Remote} ($>150 \text{ s}^{-1}$) and the possibility for both centres to bind and oxidise Fe(II), the T1_{Remote} site is not needed for oxidase activity of Cp, but might be important in increasing the overall efficiency of Fe metabolism [15]. By contrast, the T1_{PR} copper site in human Cp is redox and catalytically irrelevant, characterised by extremely high redox potential (*vide infra*) and lacking the Cys-2His ET pathway to the T2/T3 cluster. This site could be a nonfunctional vestige of gene duplication [16].

The redox potentials of the T1_{CysHis} and T1_{Remote} copper sites (E_{T1}) of human Cp were first measured by mediated redox titration in 1973 and were found to be 490 mV and 580 mV vs. NHE, respectively [17]. Later studies, however, suggested that the redox potentials of these sites are identical and equal to 448 and 434 mV in phosphate buffer in the absence and presence of Cl[−], respectively [15]. As for the T1_{PR} site, its E_{T1} -value was estimated to be very high, approximately 1000 mV vs. NHE [16]. The redox potentials of the T2 and T3 sites were found to be 491 and 415 mV respectively in phosphate buffer in the absence of Cl[−], and 539 and 482 mV respectively in the presence of Cl[−] [15].

The possibility of direct electron transfer (DET) between Cps of different origin and electrodes was the focus of investigations at different laboratories [18–20]. However, no electrochemical contact

between the enzyme and any electrode [18] had been observed or only irreversible electrochemical activity from denatured human and porcine Cp [19,20] was registered.

The only successful bioelectrochemical DET studies of the redox enzyme published in a peer-reviewed international journal were performed by Prof. Alexander I. Yaropolov with co-workers; Yaropolov was a visiting researcher at the group of Prof. Lo Gorton in the early 1990's [21]. Their collaborative work confirmed without a doubt the general possibility of DET between Cp and carbon or platinum electrodes using different electrochemical methods, such as voltammetry, potentiometry, and spectroelectrochemistry. Subsequently, one of the main thrusts of the scientific activity of Prof. Gorton was related to DET investigations of multicopper oxidases from different sources [22,23]. Well-pronounced bioelectrocatalytic reduction of O₂ by plant and fungal laccases [21,24], as well as BODs [25,26] adsorbed on graphite electrodes, was shown. However, complete inability of adsorbed Cp to accelerate electroreduction of O₂ was also observed [21,23]. In work to be reported below, we revisit the case of Cp, reporting not only new data, but also a new functional interpretation of known facts about Cp based on ideas developed by the Gorton group over the last few years.

Developments in nanotechnology, by greatly expanding the range of surfaces that can be considered as potential electrodes, created additional possibilities for fundamental electrochemical studies of redox enzymes and their application in bioelectronics [27–29]. Here we report DET studies of human Cp immobilised on bare graphite and gold (Au) electrodes, as well as on electrodes modified with carbon nanotubes (CNT) or Au nanoparticles (Au NP). We also considered thiol-modified Au surfaces. In spite of the variety of immobilisation methods used in the present studies, the previously observed inability of adsorbed Cp to catalyse electroreduction of O₂ was confirmed. Possible reasons for such anomalous behaviour of this enzyme are suggested using a modern comprehensive picture of the thermodynamics and kinetics of the mechanism of Cp function.

2. Materials and methods

2.1. Chemicals

Na₂HPO₄, KH₂PO₄, KCl, NaCl, NaF, H₂O₂, H₂SO₄, HNO₃, and NaF were obtained from Merck (Darmstadt, Germany). Single- and multi-wall carbon nanotubes (SWCNT and MWCNT, respectively), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), *n*-hydroxysuccinimide (NHS), 4-aminothiophenol (AMTP), and 6-mercapto-1-hexanol (MHOL) were from Sigma-Aldrich (St. Louis, MO, USA). 3-mercaptopropionic acid (MPA) and NaIO₄ were from Janssen Chimica (Geel, Belgium). Absolute ethanol (99.7%) was from Solveco Chemicals AB (Täby, Sweden). 1-decanethiol (DT) was obtained from Fluka (Buchs, Switzerland). Trisodium citrate-2-hydrate was from Riedel-de Haën (Seelze, Germany). All chemicals were analytical grade. Buffers were prepared with water (18 MΩ) purified with a PURELAB UHQ II system from ELGA Labwater (High Wycombe, UK). Anaerobic conditions were established using nitrogen (N₂) from AGA Gas AB (Sundbyberg, Sweden) that was bubbled through the working solutions.

2.2. Biopreparations

Human ceruloplasmin (Cp) was purchased from Sigma-Aldrich and used without further purification. The concentration of the enzyme in the stock solution was determined by the established method of Ehresmann [30]. Human serum, originated from one apparently healthy male volunteer, was prepared by a standard method known in the art and stored at +4 °C until use. For serum preparation, 9NC tubes from BD Vacutainer® (Plymouth, UK) were used.

2.3. Synthesis and characterisation of gold nanoparticles

Au NP were synthesised as described in [31]. Briefly, 50 ml of 1 mM $\text{HAuCl}_4 \times \text{H}_2\text{O}$ solution was heated to boiling temperature with stirring; then 2.96 ml of 1% trisodium citrate was added to the solution. The heating was continued for 15 min with stirring, and for a further 10 min without stirring. After cooling down to room temperature, the solution was dialysed against H_2O at the volume ratio 1:200 for 12 h in order to remove excessive citrate.

To estimate the size of the Au NP, the theoretical light absorption spectrum was generated using the Mie-scattering algorithm in the MiePlot software (<http://www.philipaven.com/mieplot.htm>). Depending on the size of NP, the shape of spectrum and absorption peak maximum is different, and the properties of theoretical spectrum should be close to the spectrum of the Au NP solution if the size of NP set up in the algorithm is the same as in the real solution. In the software the parameters were chosen as follows: refractive index of medium (water) – 1.3313847, Au sphere real – 0.1557086, Au sphere imaginary – 3.6016265 at 23 °C. The algorithm was set to *Mie* and *Qext/Qsca/Qabs v wavelength*, and the radius of the particles was set to 25 nm. The generated light absorption spectrum (*Qext* part) was compared to the spectrum of the synthesised Au NP, with the peak maximum at 530 nm, and of 25 nm achieved radius (Fig. 2A).

2.4. Electrochemical measurements

2.4.1. Cyclic voltammetry

Cyclic voltammetry (CV) was performed in an electrochemical cell of volume 20 ml containing an $\text{Ag}|\text{AgCl}|3 \text{ M KCl}$ (210 mV vs. NHE) reference electrode and a platinum mesh counter electrode using a

potentiostat/galvanostat 2059 combined with a function generator 7800 from Amel Instruments (Milano, Italy). As supporting electrolytes the following solutions were used: (i) 0.1 M phosphate buffer solution, pH 7.4; (ii) 0.1 M phosphate buffer solution, pH 7.4 with Cl^- (150 mM) or F^- (10 mM); and (iii) human serum. All potentials in the present work are given vs. NHE.

Three types of working electrodes were used in our studies: (i) disk Au electrodes purchased from Bioanalytical Systems (West Lafayette, IN, USA) with geometrical area of 0.02 cm^2 ; (ii) Au substrates manufactured in a Balzers UMS 500 P system by electron-beam deposition of 2000 Å of Au onto silicon (100) wafers (planar Au electrodes) that had been precoated with a 25-Å-thick titanium adhesion layer (Laboratory of Applied Physics, Linköping University, Sweden) with geometrical area of about 0.32 cm^2 ; and (iii) spectrographic graphite electrodes (SPGE) from Ringsdorff Werke GmbH (Bonn, Germany, type RW001, 13% porosity) with geometrical area of 0.073 cm^2 .

SPGE were polished with wet fine emery paper (Tufback Durite, P1200), rinsed thoroughly with H_2O and allowed to dry. The adsorptive roughness factor of such an electrode was estimated previously to be about 5 [32].

Both types of Au electrodes, disk and planar, were cleaned by a series of CV scans at a 100 mV s^{-1} scan rate between 0 and +1900 mV vs. NHE in 0.5 M H_2SO_4 . Immediately before usage they were rinsed thoroughly with H_2O . In addition, the disk Au electrodes were pre-cleaned by CV scans at a rate of 100 mV s^{-1} , between 0 and –1400 mV vs. NHE in 0.5 M NaOH, then polished with a DP-suspension (1 μm high performance diamond product) and an alumina de-agglomerated polishing suspension (0.1 μm , Struers, Copenhagen, Denmark), rinsed with Millipore H_2O , and sonicated in H_2O for 10 min after each polishing step. The microscopic roughness factors of Au electrodes were calculated from the charge (q_{real}) associated with the Au oxide reduction process, obtained when running a CV from 0 to 1900 mV vs. NHE in 0.5 M H_2SO_4 . The theoretical charge density (σ_{r}) associated with the reduction of the Au oxide is $390 \pm 10 \mu\text{C cm}^{-2}$ [33].

When Au disk electrodes were modified by Au NP, the electrodes were etched for 10 s in *aqua regia*-like solution produced by freshly mixing concentrated HNO_3 and KCl in a volumetric ratio of 1:3, thoroughly rinsed with H_2O , and polished with a DP-suspension to obtain as smooth a surface as possible, then rinsed with H_2O , and sonicated for 10 min.

2.4.1.1. Immobilisation of Cp on bare and thiol-modified planar gold electrodes.

For physical adsorption of Cp on the disk Au electrode, a drop of 10 μl of Cp (10 mg ml^{-1} in 10 mM phosphate buffer, pH 6.0) was placed on the Au surface. The electrode was covered to avoid evaporation and left to adsorb the protein for 3 h. For covalent binding of Cp, the Au electrode was first prepared by immersion in 10 mM 4-aminothiophenol (ethanol solution) for 4 h. The covalent binding was carried out using two different procedures (Scheme 1). In the first procedure, 2 μl of Cp (10 mg ml^{-1} in 10 mM phosphate buffer, pH 6.0) was deposited onto the monolayer-modified Au electrode. After 15 min, 4 μl of 10 mM phosphate buffer, pH 6.0, containing 35 mM NHS and 52 mM EDC, was added for covalent binding between the carboxylic groups of the protein and the amine groups of the monolayer, and left to react for 2 h (Scheme 1A). In the second procedure, 10 μl of Cp (10 mg ml^{-1} in 10 mM phosphate buffer, pH 6.0) was transferred into 200 μl of a 10 mg ml^{-1} solution of KIO_4 in water and left to react for 30 min. After that, 400 μl of 100 mM Na_2HPO_4 was added to raise the pH to 7. The monolayer-modified electrode was immersed in this solution for 1 h, to enable the reaction of the oxidised sugar residues of the protein to form a Schiff base with the primary amine present in the monolayer (Scheme 1B). Before measurement the electrodes were carefully washed with buffer.

Fig. 2. (A) The recorded and modelled spectra of 25 nm radius gold nanoparticle solution. (B) CVs of a bare Au-nanoparticle-modified gold electrode and an electrode modified with Cp in the absence and presence of 10 mM NaF (0.1 M air-saturated phosphate buffer, pH 7.4; scan rate 10 mV s^{-1} ; starting potential –1000 mV).

Scheme 1. Covalent immobilisation of Cp on the amino-modified gold surfaces.

2.4.1.2. Immobilisation of Cp on gold-nanoparticle-modified gold electrodes. First, Au electrodes were modified with Au NP: 10 μl of a solution containing NP was added on the tip of the electrode, after which the electrode was allowed to dry and then rinsed thoroughly with H_2O . Second, 10 μl of Cp (10 mg ml^{-1} in 10 mM phosphate buffer, pH 6.0) was placed on the Au surface. The electrode was covered to avoid evaporation and left to react for 90 min.

2.4.1.3. Immobilisation of Cp on bare and CNT-modified SPGEs. A volume of 10 μl of Cp solution (ca. 1 mg ml^{-1} in 5 mM phosphate buffer, pH 7.4) was placed on the cleaned bare or CNT-modified SPGE surface and allowed to adsorb for 30 min.

The nanotube-modified SPGEs were obtained as described in [34]. Briefly, a solution of SWCNT or MWCNT (10 mg ml^{-1} in 5 mM phosphate buffer, pH 7.4) was sonicated for 10 min, diluted with ethanol to a final concentration of 0.5 mg ml^{-1} ; then 10 μl of the solution was placed onto the SPGE surface and left to evaporate.

SPGE electrodes were also modified with a mixture of CNT and Cp. In this case, 10 μl of SWCNT or MWCNT (1 mg ml^{-1} in 5 mM phosphate buffer, pH 7.4) were mixed with 10 μl of Cp (1 mg ml^{-1} in 5 mM phosphate buffer, pH 7.4), shaking the solution from time to time. After 1 h, 10 μl of the mixture was placed onto the SPGE surface and left to evaporate.

2.4.2. Chronoamperometry

Chronoamperometric measurements were performed using a BAS CV-50 W voltammetric analyser (BAS, Bioanalytical Systems, West Lafayette, IN, USA) with a silver wire as a combined reference and counter electrode and the Au plate or SPGE as working electrode. The chronoamperometric current responses were registered during 10 min under aerobic and anaerobic conditions at room temperature. Three potentials were applied, viz. 350, 550, and 750 mV vs. NHE using two different supporting electrolytes: 10 mM phosphate saline buffer (PBS), pH 7.4 with 137 mM NaCl and 2.7 mM KCl, or human serum. Current responses were recorded for bare and thiol-modified Au electrodes and bare SPGE with and without immobilised Cp.

2.5. Ellipsometry measurements

The adsorption of Cp onto bare or thiol-modified Au was studied with the *in situ* ellipsometry technique, which measures the changes in polarisation state of light which is reflected at a planar surface. A thin film automated ellipsometer (type 43 603-200E, Rudolph Research, Fairfield, NJ, USA) was equipped with a xenon lamp with a fixed angle of incidence (67.8°). The light was detected at 442.9 nm employing an interference filter with ultraviolet and infrared blocking (Melles Griot, Netherlands). The Au surface was vertically mounted into a glass trapezoid cuvette (Hellma, Germany) containing 6 ml of solution, which was thermostated at 25 °C and stirred using a magnetic stirrer with a rotation speed of 325 rpm. The changes in ellipsometric angles were recorded *in situ* every 15 s. In order to determine the refractive index of the surface, a two-zone surface calibration in buffer solution was carried out prior to each measurement. When Cp was to be adsorbed on the cleaned Au plate, first a

stable baseline acquisition was done, and then enzyme from the stock solution was added to the cuvette containing 10 mM PBS buffer at pH 7.4, to a final concentration of 0.5 mg ml^{-1} . The formation of protein film was monitored for 30 min, followed by rinsing in PBS buffer for 5 min. Cp was also immobilised on a 4-aminothiophenol-modified surface, prepared by immersion of the Au plate in 10 mM ethanolic thiol solution for 4 h. The sugar residues of protein were oxidised according to the procedure described in Section 2.4.1.1, by addition of 100 μl of Cp (10 mg ml^{-1}) to 2 ml of 10 mg ml^{-1} KIO_4 water solution and a 30 min reaction. Subsequently, the whole reaction solution was added to the cuvette filled with 4 ml of 100 mM Na_2HPO_4 buffer with the thiol-modified Au surface already installed. The reaction was monitored for 1 h followed by rinsing of the cuvette for 5 min with phosphate buffer at a continuous flow of 18 ml min^{-1} , in order to monitor protein desorption.

From ellipsometric data, the absolute protein layer thickness as well as the adsorbed amount in pmol cm^{-2} were calculated using the value of 0.18 ml g^{-1} [35] as the refractive index increment with respect to change in protein concentration (dn/dc) and MW of Cp equal to 132 kDa [12].

3. Results

3.1. Electrochemistry of Cp on Au

Cyclic voltammograms (CVs) of Au NP-modified Au electrodes with physically adsorbed Cp in the presence and absence of F^- are shown in Fig. 2B. An electrochemical process at ca. 730 mV vs. NHE could be seen. This process could be assigned to the redox transformation of Cp though not with certainty. Moreover, CVs of macroscale planar bare Au disk electrodes with physically adsorbed Cp showed no redox transformation of the enzyme under either anaerobic or aerobic conditions in the potential range between 0 and 1000 mV vs. NHE at scan rates varying from 10 up to 1000 mV s^{-1} (Fig. 3A). Therefore, alternative modifications to the Au surfaces were tried in the hopes of achieving a proper enzyme orientation that would facilitate DET between copper ions of Cp and the Au electrodes.

The Au surfaces were modified with thiol monolayers providing hydrophobic, anionic-hydrophilic, cationic-hydrophilic, or charge-neutral hydrophilic surfaces. Generally, no redox transformation of the enzyme was observed when hydrophobic surfaces were made with decanethiol; when charge-neutral hydrophilic surfaces were made with 6-mercaptop-1-hexanol; or when anionic-hydrophilic surfaces were made with 3-mercaptopropionic acid. Only the covalent binding of Cp to the cationic-hydrophilic Au surface realised by forming a Schiff base between the 4-aminothiophenol (ATP) and chemisorbed Cp was successful in obtaining a DET reaction. Fig. 3B shows the CVs of an ATP-Au electrode before and after the covalent linking of the enzyme and in the absence and presence of O_2 . Obviously, the shape of the control CVs at high redox potential of the monolayer-modified electrodes of Fig. 3B is quite different from those measured after Cp attachment. A single anodic redox peak was observed with a peak potential (E_p) of about 750 mV vs. NHE (Fig. 3B, arrow). Removal of O_2 from the electrolyte solution had only a minor

Fig. 3. CVs of planar gold electrodes with immobilised Cp (0.1 M phosphate buffer, pH 7.4; scan rate — 100 mV s⁻¹). A and B represent CVs of a gold electrode with and without adsorbed Cp and a 4-amino-thiophenol-modified gold electrode with and without covalently attached Cp, respectively.

Fig. 4. Ellipsometry studies of Cp absorption on gold (0.05 M phosphate buffer, pH 7.4 containing 0.14 M of NaCl; adsorbed amount (■, curve 1); thickness (x, curve 2). (A) Time dependence of adsorption of Cp on bare gold electrode surface. Arrows indicate first protein additions and then later rinsing with buffer. (B) Time dependence of adsorption of 4-amino-thiophenol on bare and Cp on the thiol-modified gold surface. Arrows indicate addition of thiol, addition of Cp, and finally rinsing with buffer.

effect on the CVs (Fig. 3B). Theoretically, the number of electrons transferred from the electrode to the Cp molecule can range from one to six but can realistically be assumed to be in the range 1 ([25]) to 4 ([36]) or 5 (T1_{PR} is electronically isolated from the other five Cu ion sites). The corresponding concentrations of electroactive Cp on the ATP-Au surface would be in the range 1.6–7.8 pmol cm⁻² taking into account the roughness factor of the electrode of about 2. In spite of the observed DET reaction between NP- and thiol-modified Au electrodes and Cp, no catalytic wave could be detected under air-saturated condition. In addition, chronoamperometric studies of bare and ATP-modified planar Au electrodes modified and unmodified with the redox protein under N₂ and O₂, in the presence and absence of enzyme inhibitor, F⁻, confirmed without a doubt the lack of bioelectrocatalytic activity of Cp adsorbed on Au surfaces (data not shown).

3.2. Ellipsometry measurements

To confirm the presence of the protein on bare and ATP-modified planar Au surfaces, ellipsometry studies were performed (Fig. 4). It was shown that adsorption of Cp on modified and unmodified Au surfaces is a rapid process which is finished within 10–15 min. An apparent lack of adsorption reversibility and very small desorption of the protein from the Au under rinsing conditions could also be observed. The total concentration of Cp (irrespective of its electronic connection to the electrode) on the bare Au surface was calculated to be about 3.3 pmol cm⁻² and was present in a layer of absolute

thickness 50 Å (Fig. 4A). This coverage is equal to the theoretical value for a compact monolayer of Cp, which in the native conformation is roughly an equilateral triangle with side 75 Å and height in the range 40–64 Å, depending on the configuration of extending loops (Fig. 1B) [7].

The concentration of ATP on the bare Au surface was calculated to be about 700 pmol cm⁻² and with an absolute thickness of about 8 Å

Table 1

Some parameters of bare and CNT-modified SPGEs with immobilised celulopectin (air-saturated 0.1 M phosphate buffer, pH 7.4).

Parameters		Bare	SWCNT	MWCNT	SWCNT*	MWCNT*
Cathodic wave	Potential (mV)	340	335	330	365	345
	Coverage (pmol cm ⁻²)	42	214	155	124	149
	Potential (mV)	580	580	585	625	—
	Coverage (pmol cm ⁻²)	15	20	50	185	—
Anodic wave	Potential (mV)	425	410	420	450	410
	Coverage (pmol cm ⁻²)	72	425	188	209	155
	Potential (mV)	790	—	790	—	—
	Coverage (pmol cm ⁻²)	n.d.	—	18	—	—

Notes: “*” — SPGE has been modified with a mixture of CNTs and Cp; “—” — not observed; “n.d.” — not determined; the geometric area of 0.07 cm² was used for calculation.

Fig. 5. CVs of MWCNT-modified SPGE with and without Cp (0.1 M phosphate buffer, pH 7.4; scan rate 100 mV s^{-1} ; starting potential -1000 mV). A and B represent raw CVs and background-current-subtracted CVs, respectively.

(Fig. 4A), which is quite close to the theoretical values for phenol monolayer on Au [37]. The concentration of Cp on the thiol-modified Au was found to be about 1.9 pmol cm^{-2} with an absolute thickness of 21 Å (Fig. 4B). These concentrations and thicknesses also conformed to expectations.

3.3. Electrochemistry of Cp adsorbed on SPGEs

In the presence of O_2 , one well-pronounced redox process was always observed in the raw CVs of human Cp directly adsorbed on bare and CNT-modified SPGEs with a midpoint potential (E_m) of about $390 \pm 18 \text{ mV}$ (Table 1).

For simplicity, only one case is presented in this report illustrating typical CVs of Cp-modified SPGE (Fig. 5A).

For most SPGE electrodes modified with the enzyme, an additional high redox potential cathodic wave appeared with an E_p of about $600 \pm 18 \text{ mV}$. On CVs of bare and MWCNT-modified SPGE with immobilised Cp, an anodic wave also appeared resulting in E_m of about 690 mV vs. NHE (Fig. 5). After subtracting the background, current voltammetric waves were symmetrical and well-defined. Peak separations (ΔE_p) for the low and high redox potential Faradaic processes were $80 \pm 10 \text{ mV}$ and 205 mV , respectively (Fig. 5B). Calculations of the surface concentration of electroactive species (Γ) from the charge associated with both anodic and cathodic waves were performed and values are presented in Table 1. As above, assuming an electron transfer process involving one to five electrons, the concentration of electroactive Cp on bare SPGE surface (unmodified with CNT) was in the range

from 1.7 to 8.4 pmol cm^{-2} based on the low redox potential cathodic waves with E_p of 340 mV vs. NHE (Table 1), taking into account a roughness factor of the electrode equal to 5. Using the high redox potential process the coverage was in the range from 0.6 to 3.0 pmol cm^{-2} .

Since Cl^- is an important, physiologically relevant effector of the redox potentials of the T2/T3 copper cluster in Cp [15], careful investigation of bioelectrochemical behaviour of the enzyme was performed in different media, including chloride-containing buffer and human serum, using CV and chronoamperometry. The low redox potential process with E_m of 390 mV was slightly changed to the negative direction (ca. 15 mV) in the presence of 0.15 M of NaCl, whereas the high redox potential process was not affected by Cl^- .

In spite of the well-pronounced DET, no catalytic wave of O_2 electroreduction under air-saturated conditions was observed, neither when using phosphate buffers with and without Cl^- , nor when using human serum, as supporting electrolytes. It is important to emphasise, however, that peak potentials and, especially, the intensity of registered Faradaic processes strongly depended on O_2 concentration (e.g., Fig. 5). In the case of Cp-modified SPGE, removal of O_2 from the solution resulted in a significant increase of the intensity of the low potential process with an E_m shift of about 15 mV in the negative direction along with the disappearance of the high potential nonturnover signal of the enzyme (not shown). For Cp-CNT-modified SPGE, removal of O_2 from the solution resulted in almost complete disappearance of the electrochemical activity of the enzyme (Fig. 5).

4. Discussion

In the present studies, it appeared straightforward to immobilise significant amounts of Cp on Au and graphite surfaces, as verified using electrochemical and spectral techniques. It was shown previously that Cp, when adsorbed on silicon wafers with a natural layer of silicon oxide, is oriented with one of its larger sides adjoining the surface (Fig. 1B) [7]. This orientation seems also to be observed for the enzyme directly adsorbed on a bare Au surface. Because of the shape of the Cp molecule, the average thickness of the protein monolayer of 50 Å was registered, within the expected range of $64\text{--}40 \text{ Å}$ (Fig. 1B) [6]. The absolute thickness of the Cp layer on the ATP-modified Au surface was also substantial, though calculated to be less than on Au, 21 Å . This is because only about half the amount of Cp was adsorbed on this surface compared to on the bare Au. Also, there is some uncertainty associated with the specific assumptions made about several parameters that enter into our calculations, such as refractive indices of ATP and Cp films formed in two ambient media (ethanol and buffer solution).

In spite of the very high concentrations of Cp on bare Au and graphite electrodes, ATP-modified Au and CNT-modified graphite electrodes, as well as the DET reaction observed between Cp and ATP-modified Au and all graphite electrodes, even minor acceleration of O_2 electroreduction by Cp was never observed. This was the case using supporting electrolytes of different compositions and pHs, including the natural medium of this complex redox enzyme, human serum. In previous mediator-less studies of Cp, a total inability of the adsorbed enzyme to reduce O_2 was also observed [21,38]. It might be the case that the functionality of Cp is severely impaired in the surface-adsorbed state. However, it was confirmed that Cp immobilised on carbon electrodes retains approximately 50% and 30% of its activity towards adrenaline and Fe(II), respectively, compared to the homogeneous oxidation of these substrates [38]. A significant decrease in turnover numbers of enzymes in heterogeneous reactions compared to homogeneous catalysis is a quite usual situation and does not necessarily imply partial denaturation of the protein on the electrode surface. Rather, it might reflect some diffusion limitations during heterogeneous catalysis. Also, as protein flexibility is essential for proper catalysis of blue multicopper oxidases [39], any restriction to Cp movements after adsorption on the surface will most likely

influence the turnover number negatively. Indeed, it was postulated that the absence of bioelectrocatalytic activity of Cp is not related to the denaturation of the protein on the electrode surfaces, but rather reflects an ordered mechanism of Cp catalysis [40], during which an electrode cannot serve as a substrate of the enzyme [21,38]. Later, however, well-pronounced mediator-less bioelectrocatalysis for copper-containing complex redox enzymes, e.g. ascorbate oxidase [41] and copper nitrite reductase [42], was shown, even if ordered mechanisms were also suggested to explain the reduction of catalytic turnover in the heterogeneous state [43–45].

To find possible explanations for the inability of electrode-adsorbed Cp to accelerate electroreduction of O₂, one should understand which copper site of this complex redox enzyme is in DET contact with the electrode. This is not an easy task. In general it is rather difficult to observe directly the electrochemical conversion of a copper centre of blue multicopper oxidases because of the large distance between the electrode and copper sites of the proteins, as well as the high affinity of these redox enzymes to O₂. Anyhow, the electrochemical conversion of copper centre(s) of Cp immobilised on ATP-modified Au, bare graphite, and CNT-modified SPGEs, was clearly shown in the present work in agreement with previously published results on a simple surface [21,38].

Interestingly, the electrochemical behaviour of Cp adsorbed on bare SPGE is very similar to that of three-domain blue multicopper oxidases, BOx, adsorbed on the same electrodes (cf. Fig. 5 in the present study with Fig. 1 in [25]). BOx contains only one T1 site, which is the first electron acceptor in homogeneous and heterogeneous catalyses, along with the T2/T3 copper cluster responsible for O₂ reduction [25,46]. On CVs of BOx-modified SPGE, two nonturnover Faradaic signals with E_m of 690 mV and 390 mV were clearly observed under anaerobic conditions, and they are very similar in shape, intensity and E_p to those for Cp-SPGEs (Fig. 5; Table 1). Based on detailed spectroelectrochemical studies of different fungal BOxs [47], two processes with E_m of 690 mV and 390 mV were assigned to the redox transformations of the T1 site and the T2/T3 cluster of the enzyme, respectively [25]. In the case of human Cp, the E_m of the high potential Faradaic process is significantly higher than the reported redox potentials of the T1_{Cys-His} and T1_{Remote} sites of the enzyme (434–580 mV vs. NHE according to different authors [15,17]). However, this difference can be explained by a conformational transformation in the vicinity of the copper ions during adsorption of the enzyme. Moreover, a very minor change of this redox process on Cl[−] addition was registered in CV measurements, in agreement with the behaviour of the T1 sites observed during redox titration experiments [15]. Furthermore, as already shown for BOx, some values of redox potentials of the T1 sites of the blue multicopper oxidases are determined by the reductive titration procedure without electrochemical control, and reversible oxidative titrations were much smaller than the real potential of the T1 site of BOD carefully measured later on [47]. Thus, the general possibility that the nonturnover Faradaic process with E_m of 690 mV belongs to either the T1_{Cys-His} or T1_{Remote} sites of Cp should not be prematurely rejected simply because of moderate disagreement between E_m in CV measurements and reductive redox titrations. In contrast, T1_{PR} of Cp was not, in all likelihood, electrochemically detectable under conditions used in the present work because of its very high redox potential.

As for the T2 and T3 copper sites, in our opinion, it is better to operate by the term of “redox potential of the T2/T3 copper cluster ($E_{T2/T3}$)”, since these two sites form the trinuclear cluster with maximal distance between all copper ions less than 5 Å (Fig. 1A). The T2/T3 cluster of blue multicopper oxidases has many electronically and structurally different intermediates that form as the enzymatic reaction of O₂ reduction progresses, and many $E_{T2/T3}$ -values of the cluster are expected [48]. The low potential Faradaic process with E_m of 390 mV vs. NHE might be assigned as one of the redox potentials of

the T2/T3 cluster of Cp because of its strong sensitivity towards O₂ concentration. Moreover, one of the redox potentials of the cluster of blue multicopper oxidases, viz. fungal Lcs [23,48–50] and BOx [25,47], was also found to be ca. 400 mV, in agreement with the results obtained in the present study.

It should be mentioned, however, that some results of the present work contradict the hypothesis described just above that the two Faradaic processes observed for Cp should be described as the redox transformations of the T1 site and T2/T3 cluster. First, blue T1 sites of multicopper oxidases do not bind O₂, and their nonturnover Faradaic signals should not be affected by O₂ concentration. In our studies, however, the disappearance of the high redox potential process under anaerobic conditions is clearly seen (Fig. 5). Second, in accordance with redox titrations of Cp, the redox potential of the T2/T3 copper cluster should be shifted to a higher value after addition of Cl[−], an important and physiologically relevant effector of the redox potentials in human Cp [15]. In our studies, however, only a minor effect of Cl[−] on redox processes was registered. Obviously, to unambiguously determine all redox potentials of different copper sites of Cp, additional mediated and mediator-less spectroelectrochemical studies of the protein are needed. Such studies are in the scope of our further investigations.

To summarise, different immobilisation methods were exploited in our studies including nanotechnological achievements, i.e. use of Au NP and CNTs, in order to enhance surface texturing and thereby extend the range of the nature of the electrode surfaces that could be considered. In no case, however, was DET-based bioelectrocatalytic reduction of O₂ by human Cp registered. At the same time, the electrochemical behaviour of Cp adsorbed on SPGE under anaerobic conditions mimics already published results concerning BOx-modified SPGE [25], for which well-pronounced bioelectrocatalysis was recently shown [25]. Thus, based on a modern comprehensive picture of the thermodynamics and kinetics of the mechanism of Cp function, one can suggest that the bioelectrocatalytic inertness of Cp is associated with a very complicated mechanism of intramolecular ET in this complex redox protein. It is widely held nowadays that all blue multicopper oxidases, including Cp, couple the four-electron reduction of O₂ to H₂O with one-electron oxidation of substrates via a ping-pong mechanism [15,51]. However, contrary to all other blue multicopper oxidases, the intramolecular ET from the T1 site (T1_{His-Cys}, Fig. 1A) to the T2/T3 copper cluster does not occur, when only one reducing equivalent is donated to T1 coppers of Cp. This reflects a kinetic trapping behaviour of this human enzyme [15], which might be the reason for the total absence of DET-based biocatalytic activity of Cp.

Cp shows commonalities with blue multicopper oxidases in general; yet Cp appears as an interesting case for which enzymatic function is not fully understood and may offer some unique surprises which await discovery and elucidation.

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