J Biol Inorg Chem (2011) 16:209–215
DOI 10.1007/s00775-010-0717-z

ORIGINAL PAPER

Artefacts induced on *c*-type haem proteins by electrode surfaces

Patrícia M. Paes de Sousa · Sofia R. Pauleta · M. Lurdes Simões Gonçalves · Graham W. Pettigrew · Isabel Moura · José J. G. Moura · Margarida M. Correia dos Santos

Received: 30 July 2010 / Accepted: 4 October 2010 / Published online: 21 October 2010
© SBIC 2010

Abstract In this work it is demonstrated that the characterization of *c*-type haem containing proteins by electrochemical techniques needs to be cautiously performed when using pyrolytic graphite electrodes. An altered form of the cytochromes, which has a redox potential 300 mV lower than that of the native state and displays peroxidatic activity, can be induced by interaction with the pyrolytic graphite electrode. Proper control experiments need to be performed, as altered conformations of the enzymes containing *c*-type haems can show activity towards the enzyme substrate. The work was focused on the study of the activation mechanism and catalytic activity of cytochrome *c* peroxidase from *Paracoccus pantotrophus*. The results could only be interpreted with the assignment of the observed non-turnover and catalytic signals to a non-native conformation state of the electron-transferring haem. The same phenomenon was detected for Met–His monohaem cytochromes (mitochondrial cytochrome *c* and *Desulfovibrio vulgaris* cytochrome *c*-553), as well as for the bis-His multihaem cytochrome c_3 from *Desulfovibrio gigas*, showing that this effect is independent of the axial coordination of the *c*-type haem protein. Thus, the interpretation of electrochemical signals of *c*-type (multi)haem proteins at pyrolytic graphite electrodes must be carefully performed, to avoid misassignment of the signals and incorrect interpretation of catalytic intermediates.

Keywords Artefacts · Electrochemistry · *c*-type haem proteins · Peroxidases · Pyrolytic graphite electrodes

P. M. Paes de Sousa (✉) · S. R. Pauleta (✉) · I. Moura · J. J. G. Moura
Requimte, Centro de Química Fina e Biotecnologia,
Departamento de Química,
Faculdade de Ciências e Tecnologia,
Universidade Nova de Lisboa,
2829-516 Caparica, Portugal
e-mail: patricia.sousa@dq.fct.unl.pt

S. R. Pauleta
e-mail: srp@dq.fct.unl.pt

M. L. Simões Gonçalves · M. M. Correia dos Santos
Centro de Química Estrutural,
Instituto Superior Técnico,
Av. Rovisco Pais,
1049-001 Lisbon, Portugal

G. W. Pettigrew
Royal (Dick) School of Veterinary Studies,
University of Edinburgh,
Summerhall,
Edinburgh EH9 1QH, UK

Introduction

We have been investigating bacterial cytochrome *c* peroxidases (BCCPs) using electrochemical methods, as these have proved their ability to provide new and alternative insights into the complex behaviour of metalloenzymes [1]. The studies were performed by protein film voltammetry using a bare pyrolytic graphite (PG) electrode. This strategy has proved to be one of the most useful approaches for the electrochemical analysis of proteins, since it has been successful in affording electrostatically controlled adsorption of proteins without denaturation or loss of biochemical activity, and has been extensively used for direct electron transfer of enzymes [1].

BCCPs are unique systems, distinct from other peroxidases, as they have the feature of requiring an activation mechanism and having a second high-potential haem, which functions as a storage for the same electron equivalent needed to generate the radical-based compound I

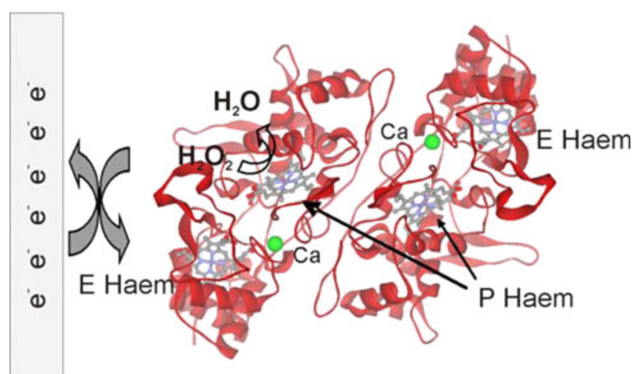

Fig. 1 *Paracoccus pantotrophus* cytochrome *c* peroxidase, highlighting the positions of the two haems relative to each other and the calcium ions (as *green spheres*). *E haem* electron-transferring haem, *P haem* peroxidatic haem

in monohaem cytochrome *c* peroxidases [2]. With the exception of the enzyme from *Nitrosomonas europaea*, all BCCPs are isolated in an inactive oxidized state and a Ca^{2+}-dependent activation mechanism is necessary to prepare the enzyme for reaction with the substrate [3].

Paracoccus pantotrophus cytochrome *c* peroxidase is a periplasmic dimeric enzyme that contains a high-potential electron-transferring haem (E haem) and a low-potential peroxidatic haem (P haem) in each monomer (Fig. 1) [2, 4, 5]. In the resting, oxidized enzyme, the iron of the P haem is low-spin, bis-His-coordinated, whereas that of the E haem is in a low-spin/high-spin thermal equilibrium and exhibits a His–Met coordination. The inactive, oxidized form of the enzyme has a closed conformation where the P haem adopts a hexacoordinate structure, hindering the peroxidatic reaction. The closed resting form of BCCP can be seen as a protective mechanism that prevents binding of hydrogen peroxide when there are insufficient electrons available for its complete reduction to water. Such a mechanism avoids the risk of both radical damage to the haem group and release of damaging hydroxyl radicals into the surroundings [3].

The entry of one electron to the high-potential haem (mixed-valence state) initiates the activation mechanism, which involves a series of spin-state and structural changes that are more striking around the P haem [6, 7]. In the active mixed-valence form, the P haem becomes high-spin pentacoordinated, providing access to the substrate to bind to the catalytic centre. It was shown that Ca^{2+} is essential for the conversion of the mixed-valence enzyme into the activated state, in which substrate has access to the P haem [8–10].

The two haem moieties have very different electrochemical and biochemical properties and the redox transitions have been studied using different techniques, such as visible spectroscopy, EPR spectroscopy, and NMR spectroscopy [7–10]. Redox potentials of +226 and −200/−100 mV versus the standard hydrogen electrode (SHE) were determined, in the presence of calcium at pH 7.5, from a potentiometric titration for the E haem and the P haem, respectively [7].

Our results show that the interaction with bare PG electrodes produces a non-native form of the enzyme that also displays peroxidatic activity. The same phenomenon was observed for the Met–His monohaem cytochromes, mitochondrial cytochrome *c* and *Desulfovibrio vulgaris* cytochrome *c*-553, as well as for the bis-His multihaem cytochrome c_3 from *Desulfovibrio gigas*.

Materials and methods

Protein purification

Paracoccus pantotrophus cytochrome *c* peroxidase, *D. vulgaris* cytochrome *c*-553 and *D. gigas* cytochrome c_3 were isolated and purified as described before [2, 11–13]. Horse heart cytochrome *c* was obtained from Sigma and used without any further purification. The concentration of the proteins was determined spectrophotometrically using the following extinction coefficients: $\varepsilon_{409,\mathrm{ox}} = 250$ mM^{-1} cm^{-1}, $\varepsilon_{553,\mathrm{red}} = 23.4$ mM^{-1} cm^{-1}, $\varepsilon_{552,\mathrm{red}} = 128.5$ mM^{-1} cm^{-1} and $\varepsilon_{550,\mathrm{red}} = 29.5$ mM^{-1} cm^{-1} for BCCP, cytochrome *c*-553, cytochrome c_3 and horse cytochrome *c*, respectively [2, 11–13].

Procedures

Voltammetric measurements were performed using an Autolab PSTAT10 potentiostat/galvanostat from ECO Chemie (Utrecht, The Netherlands) as the source of the applied potential and as the measuring device. The whole system was controlled and data were analysed with the GPES software package from ECO Chemie. The scan rate varied between 5 and 2,000 mV s^{-1}. The differential pulse (DP) voltammograms were obtained with an amplitude of 50 mV and a pulse width of 20 ms. A conventional three-electrode configuration cell was used, with a platinum auxiliary electrode and an Ag/AgCl reference electrode (+205 mV vs. SHE). Throughout the paper, all potential values are referred to the SHE and are affected by an experimental error of 5–10 mV. The working electrode was a basal plane PG disk electrode (reference ACSF01315, from GE Quartz Europe).

Electrode preparation

Before each experiment, the PG electrode was polished by hand on a polishing cloth (Buehler 40-7212) using a water/

alumina (0.05 μm) slurry (Buehler 40-6365-006) and rinsed well with Milli-Q water. The use of different particle size alumina slurries and/or sonication of the electrode had no influence on the results obtained. A 4-μl drop of working solution (containing the protein) was deposited on the electrode surface and left to dry at room temperature for 30 min.

Electrolyte and working solutions

The supporting electrolyte and protein solutions contained 10 mM phosphate buffer pH 7.0, 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (Hepes) buffer pH 7.5 or 20 mM tris(hydroxymethyl)aminomethane–HCl buffer pH 7.6 and 0.1 M NaCl, depending on the experiment. The concentrations of the proteins in the working solutions were typically around 100 μM. The results obtained with lower concentrations of BCCP (down to 1 μM) showed no differences.

In the experiments with BCCP with addition of calcium ions, 1 mM $CaCl_2$ was present both in the electrolyte solution and in the working solution. For assays with prereduced BCCP, a 125 μM BCCP solution containing 10 mM Hepes buffer, 10 mM sodium ascorbate, 100 μM 3,6-diaminodurol and 1 mM $CaCl_2$ (when in use) was prepared and incubated at 20 °C for 30 min [7], before a 4-μl drop was adsorbed at the electrode surface. For assays in the absence of calcium ions, the enzyme was incubated for 2 h with 2 mM ethylene glycol bis(2-aminoethyl ether)tetraacetic acid (EGTA), which was then removed by centrifugation at 14,000 rpm with a Millipore YM-10 Microcon. The process was repeated twice and the sample was then used with 2 mM EGTA present both in the electrolyte solution and in the working solution.

For assays with cyanide, concentrations between 5 μM and 1.5 mM were added to the electrolyte solution. All solutions were deaerated for 30 min with argon and all measurements were performed in a temperature-controlled room at 20 ± 1 °C. Assays with BCCP were performed both inside and outside a glove box and no differences were detected.

The turnover number (k_{cat}) was calculated according to $k_{cat} = i_{cat}/(nFA\Gamma)$, where i_{cat} is the catalytic current, A the area of the electrode and Γ the surface coverage, which is determined from the charge associated with the non-turnover signal.

Results and discussion

The electrochemical behaviour of *P. pantotrophus* cytochrome *c* peroxidase was analysed at a PG electrode by protein film voltammetry. The electrochemical experiments were carefully planned, taking into account all the known information concerning the activation and catalytic mechanisms of BCCP. Assays were performed with both as-isolated and mixed-valence BCCP, in the presence and absence of calcium ions (to allow or block the activation mechanism), and also in the absence and presence of a known inhibitor, cyanide [6].

In non-turnover conditions and in the presence of calcium ions only one redox signal was observed in the cyclic voltammograms of BCCP (Fig. 2a), corresponding to a reversible one-electron-transfer reaction with $E^{0\prime} = -126$ mV vs. SHE, pH 7.0.

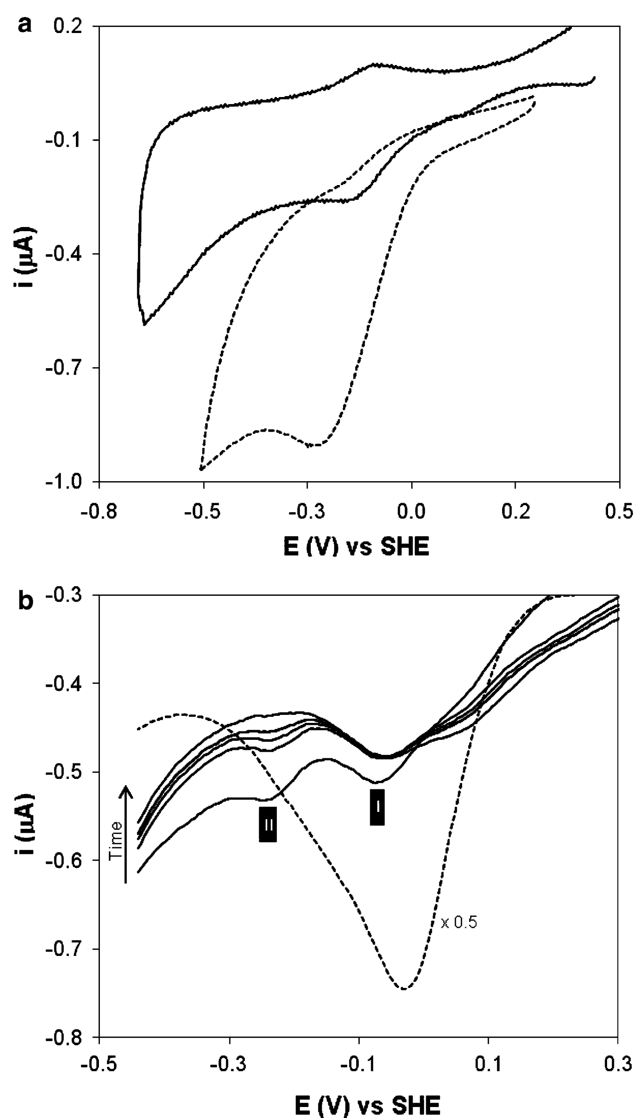

Fig. 2 Cyclic (**a**) and differential pulse (**b**) voltammograms of *Paracoccus pantotrophus* cytochrome c peroxidase adsorbed at a pyrolytic graphite electrode, in the absence (*continuous line*, 100 mV s^{-1}, baseline-corrected) and in the presence (*dashed line*, 20 mV s^{-1}) of 500 μM H_2O_2, pH 7.5. Ca^{2+} (1 mM) was present in the sample and in the electrolyte. *SHE* standard hydrogen electrode

In the presence of hydrogen peroxide, a catalytic wave is observed at a potential similar to the signal without substrate, which is consistent with a one-electron redox process (Fig. 2a). However, the first scans of DP voltammograms showed two signals with $E^{0\prime}$ of −102 mV (I) and −267 mV (II) vs. SHE, pH 7.5 (Fig. 2b). The current of peak I is 3 times higher than that of peak II. In the following few scans, peak II decreases until it completely disappears, whereas peak I does not change. Therefore, the signal observed in cyclic voltammetry must correspond to the same redox process as signal I.

Addition of hydrogen peroxide revealed catalytic activity for DP voltammograms at a potential similar to that of peak I (Fig. 2b). No other signals were detected, either in cyclic voltammetry or in DP voltammetry, for potentials as positive as 800 mV, either in the absence or in the presence of hydrogen peroxide.

Since BCCP has a high potential (+200 mV, Met–His E haem) and a low potential (−100/–200 mV, bis-His P haem) redox centre [7], the detection of two negative potential peaks in non-turnover conditions was not expected.

To identify the nature of these two signals, a series of experiments were performed and it was observed that:

1. In the absence of calcium ions, i.e. when the enzyme is not active, DP voltammograms showed both peaks with equivalent peak currents, which persist in the following scans; upon H_2O_2 addition, the magnitude of the DP voltammetry and the cyclic voltammetry electrocatalytic signals was the same as that observed in the presence of calcium ions.
2. When the E haem of the enzyme is prereduced with sodium ascorbate (mixed-valence state), only peak I is observed using both voltammetric techniques.
3. The addition of cyanide has no effect on signal I, i.e. neither its current nor its potential is altered in the presence of the inhibitor, and the catalytic activity is maintained.

According to these results, signal II must correspond to an entity that is changing with time. In the case of BCCP, it can only be attributed to the reduction of the hexacoordinated, non-activated P haem, which becomes pentacoordinated with the reduction of the E haem, in the presence of calcium ions. This is in accordance with the fact that, upon reduction with sodium ascorbate, the voltammogram of the mixed-valence enzyme only exhibited the peak corresponding to signal I. The pentacoordinated form of the P haem cannot correspond to signal I, since signal I and signal II were independent, i.e. the intensity of signal I is not affected either by the decrease of signal II or by its disappearance.

No other signals were detected and, therefore, the pentacoordinated form is silent in our electrochemical experiments. A possible explanation for the absence of signal for the pentacoordinated form is an unfavourable interaction with the electrode surface, resulting from the structural changes that occurred during the activation mechanism. Thus, signal I can only be attributed to an altered form of the E haem, which has a 340 mV decreased redox potential and maintains the peroxidatic activity in the absence of calcium ions.

The identification of signal I as an altered form of the E haem is strengthened by the following facts:

1. Met–His-coordinated *c*-type cytochromes display, in the same electrode, not only the signal due to the native state but also a more negative peak, which in some cases, as for horse cytochrome *c*, is the main signal when the protein is immobilized (Fig. 3a, b), as other authors have observed [14, 15].
2. These cytochromes display catalytic activity towards hydrogen peroxide under these conditions (Fig. 3a, b).
3. The shapes of the electrocatalytic waves and the turnover numbers of mitochondrial cytochrome *c* ($5 \pm 2\ s^{-1}$), *D. vulgaris* cytochrome *c*-553 ($7 \pm 2\ s^{-1}$) and *P. pantotrophus* cytochrome *c* peroxidase ($10 \pm 2\ s^{-1}$) are similar.

As to the nature of this non-native state of the E haem, other authors have provided evidence that Met–His *c*-type cytochromes undergo structural distortions in the presence of moderate electric fields that lead to the lost of the distal Met ligand [16, 17]. Furthermore, it is known that in BCCPs the E haem is in a low-spin/high-spin thermal equilibrium in the oxidized form, which may favour the axial Met displacement (Met289, in the case of *P. pantotrophus* [5]). This new conformation state, with the axial Met site either vacant or occupied by other ligands, has a redox potential 300 mV lower than that of its native state [16, 17]. In the case of mitochondrial cytochrome *c*, this state and its peroxidatic activity have biological relevance in the apoptotic cycle, and it has been proposed that the switch to this form occurs upon interaction with the charged lipid cardiolipin [18]. However, in the case of BCCPs, there is no evidence that this form is biologically relevant.

The formation of altered forms at PG electrodes has been proposed for *c*-type cytochromes, with either Met–His- or bis-His-coordinated haems [14, 19]. In our conditions, when *D. gigas* cytochrome c_3 is adsorbed onto a PG electrode it displays a signal with two peaks with peak potentials of −230 and −320 mV vs. SHE, in accordance with the values reported in the literature [12]. However, after a few scans only the more positive peak persists

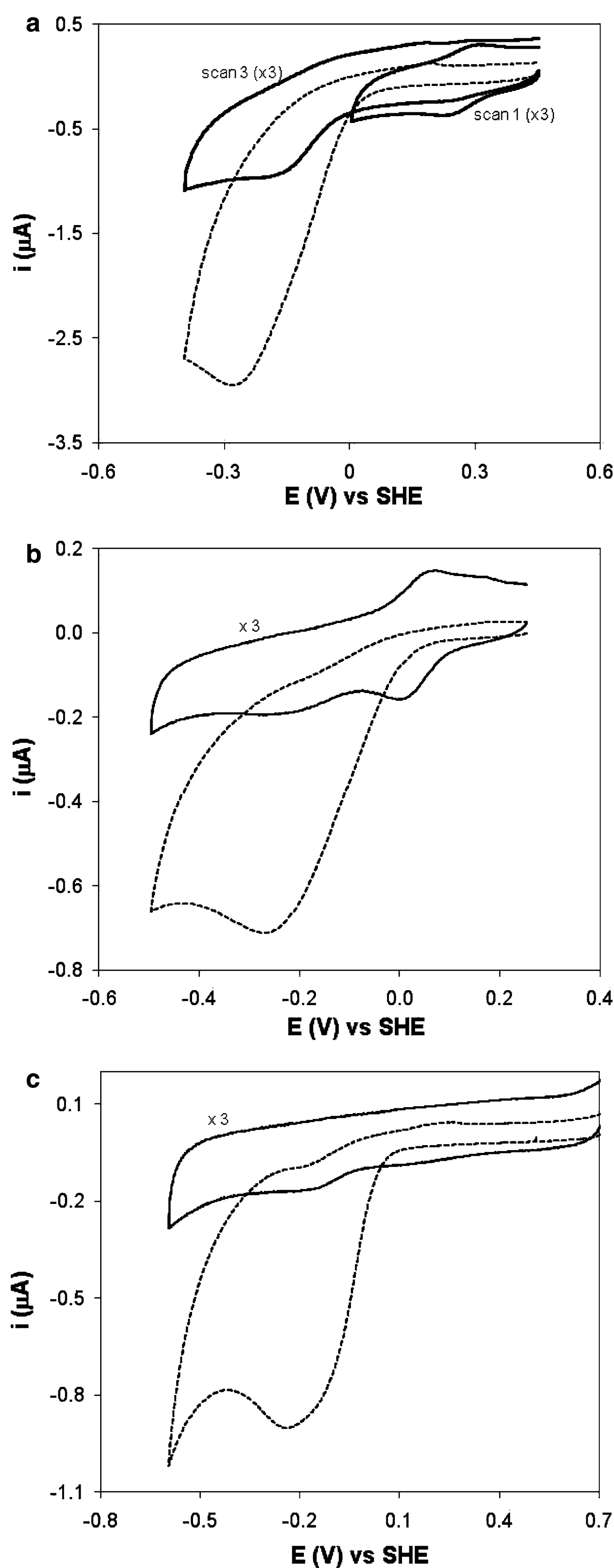

Fig. 3 Cyclic voltammograms ($v = 20$ mV s^{-1}) of horse heart cytochrome *c* (**a**), *Desulfovibrio vulgaris* cytochrome *c*-553 (**b**) and *Desulfovibrio gigas* cytochrome c_3 (**c**), in the absence (*continuous line*) and in the presence (*dashed line*) of 500 µM H_2O_2, pH 7.6

(Fig. 3c). More importantly, although not displaying a distinct identifiable altered signal, cytochrome c_3 also shows peroxidatic activity in these conditions (Fig. 3c). This peroxidatic activity is similar in shape and magnitude (10 ± 2 s^{-1}) to that of Met–His cytochromes *c* and thus can only be interpreted by the formation of an altered form. This catalytic event occurs at −34 mV, a potential more positive than that observed for the Met–His cytochromes *c* and BCCP, reflecting the different axial coordination and pocket environment of the haem.

When high-spin forms of cytochrome c_3 are induced at very low pH [20], a more intense response to H_2O_2 is observed (46 ± 2 s^{-1}, Fig. 4a). The catalytic event at low pH is more intense and at higher potential values (+190 mV) (Fig. 4b), which indicates that at neutral pH there is an equilibrium between altered (high-spin form induced by the interaction with the PG electrode) and native forms, whereas at low pH only the fully high-spin form, obtained by protonation of the distal His, interacts with the electrode. Indeed, this high-spin form of cytochrome c_3 mimics the catalytic haem of peroxidases (pentacoordinated haem with an axial His) and, similarly to the potential of compound I of yeast cytochrome *c* peroxidase [21] and the catalytic potential of the natively active *N. europaea* cytochrome *c* peroxidase [22], its catalytic potential is positive.

It is important to point out that, for all the proteins tested, independent assays gave rise to catalytic signals similar in shape and current magnitude. Moreover, successive scans always led to a decrease of the catalytic signal until the initial non-turnover voltammogram was restored, with the exception of cytochrome c_3 at pH 1.

In the light of the results presented here, the electrochemical behaviour reported for other BCCPs should be reanalyzed. In the case of *Pseudomonas aeruginosa* cytochrome *c* peroxidase, Becker et al. [23] observed two signals, −76 mV vs. SHE at pH 6 and −234 mV vs. SHE at pH 7, assigned to the pentacoordinated and hexacoordinated P haem, respectively [23]. On the basis of our observations, another explanation is possible, i.e. the more positive signal might correspond to the pentacoordinated P haem, since a decrease in peak current and activity was observed in the presence of cyanide, but the other peak could be assigned to the altered E haem. In contrast, in the case of *N. europaea* cytochrome *c* peroxidase, when the enzyme was adsorbed at a PG electrode only in catalytic conditions was a signal observed, a catalytic wave with a midpoint potential higher than 500 mV vs. SHE [22]. This indicates that a high electroactive coverage is not favoured by the surface characteristics of *N. europaea* cytochrome *c* peroxidase and thus the altered E haem conformation is not formed, enabling the study of the enzyme mechanism.

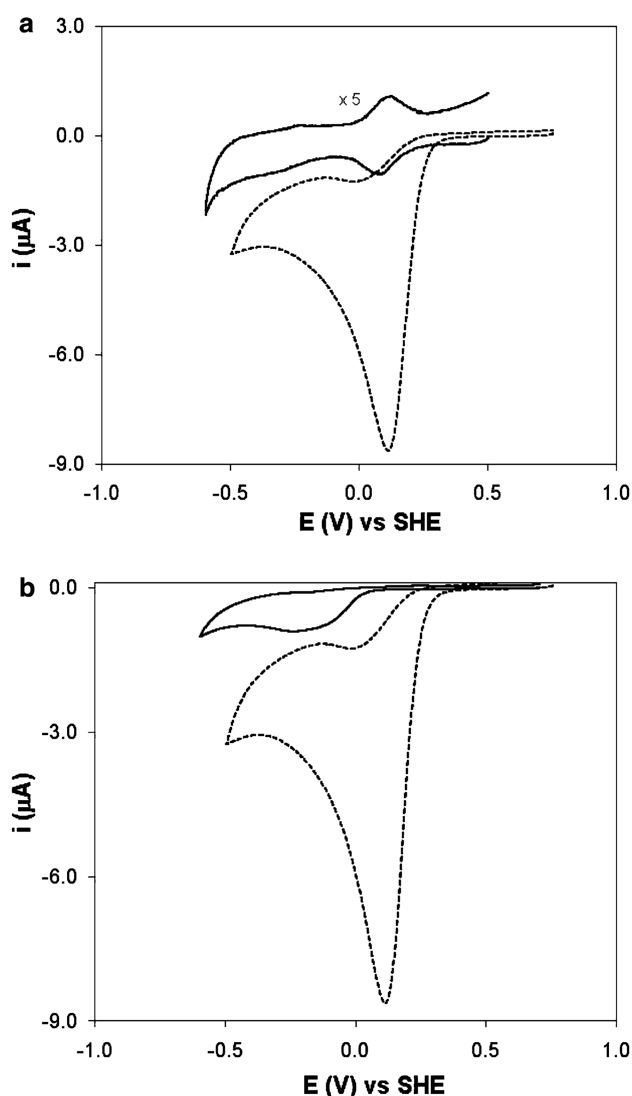

Fig. 4 Cyclic voltammograms ($v = 20$ mV s^{-1}) of *D. gigas* cytochrome c_3 **a** in the absence (*continuous line*) and presence (*dashed line*) of 500 µM H_2O_2 at pH 1.3 and **b** in the presence of 500 µM H_2O_2 at pH 7.6 (*continuous line*) and in the presence of 500 µM H_2O_2 at pH 1.3 (*dashed line*)

Finally, it is important to point out that the effects reported here for *c*-type haem containing proteins, adsorbed directly on bare PG electrodes, may occur at other electrode surfaces usually considered gentle to the proteins, such as self-assembled-monolayer-modified metal electrodes [24]. This may be the case for the results recently reported for the dihaem cytochrome c_4 [25], which could have a distinct interpretation taking into account the results presented in this work.

Conclusions

This work demonstrates that the interaction of *c*-type haem containing proteins with bare PG electrodes can induce altered forms of the haems, which have a redox potential approximately 300 mV lower than that of the native state and display peroxidatic activity. This phenomenon was observed for the Met–His monohaem mitochondrial cytochrome *c* and *D. vulgaris* cytochrome *c*-553, as well as, for the bis-His multihaem *D. gigas* cytochrome c_3, demonstrating that this effect is independent of the axial coordination of the *c*-type haem protein. The effect was also detected for the E haem of *P. pantotrophus* cytochrome *c* peroxidase, showing that altered conformations of enzymes containing *c*-type haems can have activity towards the enzyme substrate, and this can lead to a serious misinterpretation of the results.

In the last decade, several strategies have been proposed and used to obtain electrode surfaces for direct electron transfer of proteins, which may simply consist of mechanical polishing or electrochemical treatment of the electrode surface, or may be based on more elaborate procedures, such as coating the surface with nanoparticles or its chemical functionalization with a self-assembled monolayer. However, adsorption of the protein onto a freshly polished PG electrode has been one of the most used strategies over the years and therefore it is extremely important to be aware of the artefacts that may occur at this type of electrode surface.

Acknowledgment P.M.P.S. would like to thank the Ciência 2007 (FCT-MCTES) programme.

References

1. Léger C, Bertrand P (2008) Chem Rev 108:2379
2. Goodhew CF, Wilson IBH, Hunter DJB, Pettigrew GW (1990) Biochem J 271:707
3. Pettigrew GW, Echalier A, Pauleta SR (2006) J Inorg Biochem 100:551
4. Pettigrew GW (1991) Biochim Biophys Acta 1058:25
5. Echalier A, Goodhew CF, Pettigrew GW, Fülöp V (2006) Structure 14:107
6. Gilmour R, Goodhew CF, Pettigrew GW, Prazeres S, Moura I, Moura JJG (1993) Biochem J 294:745
7. Gilmour R, Goodhew CF, Pettigrew GW, Prazeres S, Moura I, Moura JJG (1994) Biochem J 300:907
8. Prazeres S, Moura I, Moura JJG, Gilmour R, Goodhew CF, Pettigrew GW (1993) Magn Reson Chem 31:S68
9. Prazeres S, Moura JJG, Moura I, Gilmour R, Goodhew CF, Pettigrew GW, Ravi N, Huynh BH (1995) J Biol Chem 270:24264
10. Gilmour R, Prazeres S, McGinnity DF, Goodhew CF, Moura JJG, Moura I, Pettigrew GW (1995) Eur J Biochem 234:878
11. Fauque G, Moura JJG, Besson S, Saraiva LM, Moura I (1992) Oceanis 18:211
12. Coutinho IB, Xavier AV (1994) Methods Enzymol 243:119
13. Bowden EF, Hawkridge FM, Chlebowski JF, Bancroft EE, Thorpe C, Blount HN (1982) J Am Chem Soc 104:7641
14. Ye T, Kaur R, Senguen FT, Michel LV, Bren KL, Elliott SJ (2008) J Am Chem Soc 130:6682

15. Casalini S, Battistuzzi G, Borsari M, Bortolotti CA, Ranieri A, Sola M (2008) J Phys Chem B 112:1555
16. Wackerbarth H, Hildebrandt P (2003) Chemphyschem 4:714
17. De Biase PM, Paggi DA, Doctorovich F, Hildebrandt P, Estrin DA, Murgida DH, Marti MA (2009) J Am Chem Soc 131: 16248
18. Belikova NA, Vladimirov YA, Osipov AN, Kapralov AA, Tyurin VA, Potapovich MV, Basova LV, Peterson J, Kurnikov IV, Kagan VE (2006) Biochemistry 45:4998
19. Haladjian J, Draoui K, Bianco P (1991) Electrochim Acta 36:1423
20. Santos M, Correia dos Santos MM, Simões Gonçalves ML, Costa C, Romão JC, Moura JJG (2006) J Inorg Biochem 100:2009
21. Mondal MS, Fuller HA, Armstrong FA (1996) J Am Chem Soc 118:263
22. Bradley AL, Chobot SE, Arciero DM, Hooper AB, Elliott SJ (2004) J Biol Chem 279:13297
23. Becker CF, Watmough NJ, Elliott SJ (2009) Biochemistry 48:87
24. Murgida DH, Hildebrandt P (2004) Acc Chem Res 37:854
25. Chi Q, Zhang J, Arslan T, Borg L, Pedersen GW, Christensen HEM, Nazmudtinov RR, Ulstrup J (2010) J Phys Chem B 114:5617