COMMUNICATION

ChemComm
www.rsc.org/chemcomm

The oxidation of cytochrome c in nonaqueous solvents

Clare Grealis and Edmond Magner*

Materials and Surface Science Institute and Department of Chemical and Environmental Sciences, University of Limerick, Plassey, Co. Limerick, Ireland. E-mail: Edmond.Magner@ul.ie

Received (in Cambridge, UK) 6th February 2002, Accepted 5th March 2002
First published as an Advance Article on the web 15th March 2002

Cytochrome c was adsorbed readily and remained electroactive, with a redox potential of 32 mV, on TiO_2 films placed on SnO_2; similar behaviour was observed in glycerol, whereas in acetonitrile, irreversible oxidation occurred at a potential of 800 mV.

The discovery that enzymes are catalytically active in nonaqueous media, and exhibit properties which can be markedly different to those obtained in aqueous solution, has generated considerable interest in the study of enzymes in such media.[1,2] Examples of some of the changes that have been observed include novel enantio- and regioselectivity, properties that are particularly attractive in preparative organic synthesis.[1] Similarly, the use of enzymes as active components of biosensors in nonaqueous solvents,[3,4] in particular for the detection of water insoluble analytes, is of interest. Extensive studies on the properties of enzymes in nonaqueous solvents have been carried out. For example, the structures of enzymes are not significantly perturbed when placed in nonaqueous solvents.[5,6] While properties such as enantioselectivity are highly desirable, a drawback in using enzymes in nonaqueous solvents lies in the reduced rates of reaction observed in comparison to those in aqueous buffer. An understanding of the reasons for such decreases is beginning to develop, while strategies which significantly increase the rates of reaction in nonaqueous solvents have been reported.[6–8]

Much of the work to date on nonaqueous enzymology has focused on hydrolases, with far less attention paid to oxidoreductases. The use of redox enzymes in nonaqueous solutions is hampered by the requirement for an exogeneous oxidant, usually in the form of a peroxide, to produce a higher oxidation state (*e.g.* compound I in peroxidases) which is the catalytically active form of the redox enzyme, oxidising the substrate to product. In order to achieve reasonable rates of reaction, typical concentrations of peroxide lie in the millimolar range, levels which quickly deactivate the enzyme and greatly reduce the turnover number of the catalyst.[9] A more satisfactory approach involves the controlled addition of oxidising equivalents, an approach which has been shown to substantially increase the yield of product and the catalytic turnover number.[10] Ultimately, it would be desirable to utilise an electrochemical approach, where the oxidation state of the enzyme could be precisely controlled. For such an approach to be successful, it is first of all necessary to quantify any changes in the electrochemical properties that result on placing an enzyme in a nonaqueous solvent.

The redox protein, cytochrome c has been well characterised electrochemically.[11] When coupled with the fact that the haem is slightly exposed to the external solution, cytochrome c is an attractive candidate for the study of the effect of the solvent on the electrochemical properties of redox proteins. However, like most enzymes and proteins, cytochrome c is insoluble in virtually all non-aqueous solvents, rendering direct oxidation or reduction in an electrochemical cell impossible. To examine the native protein, an alternative approach involving binding the protein to the surface of an electrode, is required.[7]

Cass *et al.*[12] have used films of nanoporous TiO_2 to immobilise cytochrome c. Cytochrome c (horse heart) was adsorbed onto a TiO_2 modified SnO_2 electrode.[13,14]† Over the potential range examined, TiO_2 is insulating[14] and serves only as a support for the protein. Fig. 1A shows a typical cyclic voltammogram obtained for such a modified electrode. A redox potential of 32 ± 5 mV *vs.* Ag/AgCl (n = 20 electrodes) was obtained, in good agreement with the literature value of 42 mV s^{-1}.[11] Quasi-reversible behaviour was observed with a peak separation (ΔE) of 24 mV. The response was found to be stable with there being no deviation from the initial response after ten sequential scans. These results demonstrate that TiO_2 adsorbs cytochrome c in an orientation which permits electron transfer to occur to and from the SnO_2 electrode.‡

Fig. 2A shows a cyclic voltammogram of cytochrome c on a TiO_2 film in 95% acetonitrile. In comparison with Fig. 1A the oxidation peak potential of cytochrome c has increased from 50 to 820 ± 20 mV (n = 20 electrodes). No current peak could be discerned on the reverse scan, indicating that the response was irreversible. No faradaic response was obtained in dry acetoni-

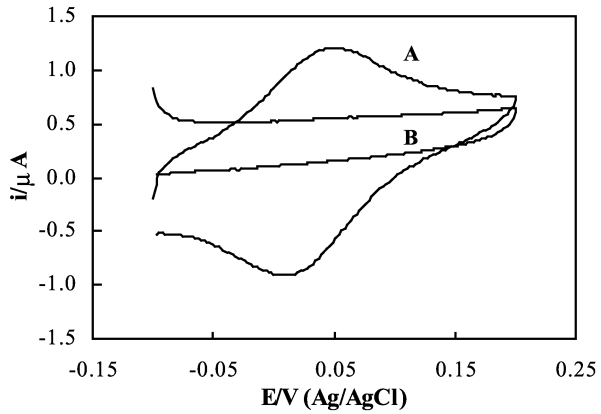

Fig. 1 Cyclic voltammogram of cytochrome c (**A**) and apocytochrome c (**B**) adsorbed onto TiO_2. Conditions: 10 mM phosphate, pH 7.0, scan rate of 50 mV s^{-1}.

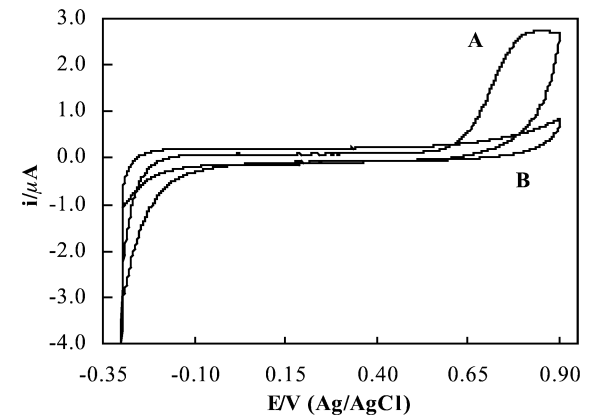

Fig. 2 Cyclic voltammogram of cytochrome c (**A**) and apocytochrome c (**B**) adsorbed onto TiO_2. Conditions: 0.1 M tetramethylammonium hexafluorophosphate in acetonitrile (5% 10 mM phosphate buffer, pH 7.0), scan rate of 20 mV s^{-1}.

DOI: 10.1039/b201259f

trile. Such a requirement for exogeneous water has been observed with a number of enzymes and can be explained by their need to possess a certain degree of hydration for catalysis to proceed.[17] In order to determine the origin of the observed redox process, apocytochrome c[18] was adsorbed onto TiO_2. On scanning the potential between −100 and 200 mV, no faradaic current was observed for the adsorbed apocytochrome c (Fig. 1B), clearly indicating that all of the haem had been removed.

When the apocytochrome c modified electrode was placed in acetonitrile (Fig. 2B), no oxidation peak was detected over the potential range 0–1000 mV. The absence of a faradaic current on removal of the haem demonstrated that it is the haem group which undergoes oxidation and not an amino acid residue.

The Raman spectrum (Fig. 3, lower spectrum) of cytochrome c adsorbed onto TiO_2 was similar to that reported by Hildebrandt *et al.*[19] for cytochrome c adsorbed onto Ag. For example, the spin marker bands ν_{11}, ν_{19} and ν_{10} at 1543, 1583 and 1635 cm^{-1}, respectively, occur at similar positions and indicate that the haem exists in the low spin state on TiO_2, as is the case in aqueous solution.[19] On exposing the electrode to acetonitrile, no changes were observed in the Raman spectrum. When the electrode was then placed in aqueous buffer, the cyclic voltammogram obtained was identical to that prior to immersion in acetonitrile. The visible spectrum of the adsorbed protein in acetonitrile was indistinguishable from the spectrum of the protein in aqueous buffer, indicating that no gross structural changes to the haem environment had occurred. These results demonstrate that exposure to acetonitrile does not perturb the environment of the haem. On oxidation of the adsorbed protein in acetonitrile, the Soret peak at 410 nm is completely removed while no peaks were observed in the Raman spectrum, over the range 900–1700 cm^{-1} (Fig. 3, upper spectrum). These changes in the visible and Raman spectra demonstrate that the haem itself has been oxidised and that all of the adsorbed protein is electroactive. The shift in oxidation peak potential may be due to slow electron transfer kinetics or to a significant increase in $E°$. At present it is not possible to distinguish between either of these possibilities. Note that by comparison, relatively small shifts in the redox potential of the haem peptide microperoxidase have been reported in dimethyl sulfoxide[20] (+115 mV), acetonitrile[21] (+60 mV) and ethanol[20] (+66 mV), while the redox potential of hemin in acetonitrile was unchanged.[22] Using cytochrome c adsorbed onto TiO_2, no faradaic response was obtained in ethanol nor in DMSO, indicating that the protein was not electroactive in these solvents. In glycerol, a reversible response was obtained with an $E°$ similar to that in buffer, while no response was obtained in propanol or formamide. Given the results obtained here, with an unaltered value for $E°$ in glycerol, the high oxidation peak potentials in acetonitrile, and the absence of a faradaic response in solvents such as propanol, it is clear that the solvent critically affects the electrochemical properties of cytochrome c. These results demonstrate that the successful use of redox proteins and enzymes in nonaqueous solvents requires detailed knowledge of their electrochemical properties in such solvents.

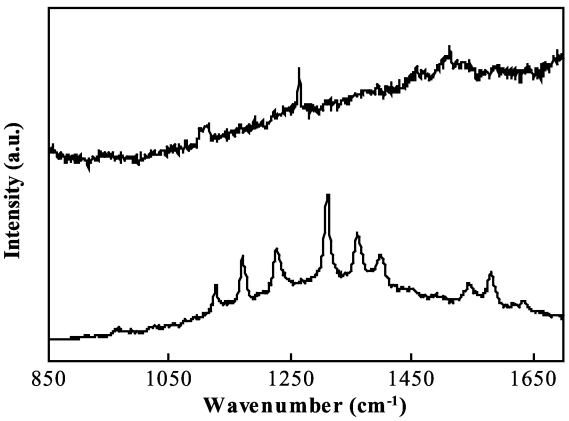

Fig. 3 Raman spectrum of cytochrome c on TiO_2 in aqueous solution (lower), and in acetonitrile (upper, after oxidation).§

This research was funded by Enterprise Ireland (SC/1998/414), MediSense, Inc., and the University of Limerick Foundation. Discussions with Professor Gabriel Barletta are gratefully appreciated.

Notes and references

† Electrodes were prepared as described in reference 13. In order to make thinner layers of TiO_2, 100 μL of 1% Tween was added to the paste to give a final volume of 200 μL.

‡ Two alternative approaches, utilising colloidal gold[15] and self assembled monolayers of alkanethiols[16] to attach cytochrome c onto gold electrodes were attempted. No detectable electrochemical response was observed with the former, while with the latter approach the potential ranges, over which the alkanethiols were not oxidized/reduced, were too narrow.

§ Resonance Raman spectra (spectral width of ~9 cm^{-1}) were obtained on a Jobin Yvon instrument (LABRAM 1/168 IM) using a Uniphase Model 2010 Ar^+ laser as the excitation source, λ_{ex} 514.5 nm.

1 A. M. Klibanov, *Chemtech*, 1996, 354.
2 M. N. Gupta, *Eur. J. Biochem.*, 1992, **203**, 25.
3 P. Julicher, L. Haalck, M. Meusel, K. Camman and F. Spener, *Anal. Chem.*, 1998, **70**, 3362.
4 S. Kröger, S. J. Setford and A. P. F. Turner, *Anal. Chem.*, 1998, **70**, 5047.
5 P. A. Fitzpatrick, A. C. U. Steinmetz, D. Ringe and A. M. Klibanov, *Proc. Natl. Acad. Sci. USA*, 1993, **90**, 8653.
6 P. P. Wangikar, P. C. Michels, D. S. Clark and J. S. Dordick, *J. Am. Chem. Soc.*, 1997, **119**, 70.
7 K. Griebenow, Y. D. Laureano, A. M. Santos, I. M. Clemente, L. Rodríguez, M. W. Vidal and G. L. Barletta, *J. Am. Chem. Soc.*, 1999, **121**, 8157.
8 J. Partridge, P. J. Halling and B. D. Moore, *Chem. Commun.*, 1998, 841.
9 E. Magner and A. M. Klibanov, *Biotech. Bioeng.*, 1995, **46**, 175.
10 E. Kiljunen and L. T. Kanerva, *J. Mol. Catal. B: Enzymatic*, 2000, **9**, 163.
11 F. A. Armstrong, H. A. O. Hill and N. J. Walton, *Acc. Chem. Res.*, 1988, **21**, 407.
12 E. Topoglidis, A. E. G. Cass, G. Gilardi, S. Sadeghi, N. Beaumount and J. R. Durant, *Anal. Chem.*, 1998, **70**, 5111.
13 P. Yeh and T. Kuwana, *Chem. Lett.*, 1977, 1145–1148.
14 A. Topoglidis, T. Lutz, R. Willis, C. Barnett, A. E. G. Cass and J. R. Durrant, *Faraday Discuss.*, 2000, **116**, 35.
15 A. L. Crumbliss, S. C. Perine, J. Stonehuerer, K. Turbergen, J. Khao, R. W. Henkens and J. P. O'Daly, *Biotechnol. Bioeng.*, 1992, **40**, 483.
16 J. M. Cooper, K. R. Greenough and C. J. McNeil, *J. Electroanal. Chem.*, 1993, **347**, 267.
17 A. Zaks and A. M. Klibanov, *J. Biol. Chem.*, 1988, **263**, 8017.
18 W. R. Fisher, H. Taniuchi and C. B. Antinsen, *J. Biol. Chem.*, 1978, **248**, 3188.
19 P. Hildebrandt and M. Stockburger, *J. Phys. Chem.*, 1986, **90**, 6017.
20 P. A. Mabrouk, *Anal. Chim. Acta*, 1995, **307**, 245.
21 A. N. J. Moore, E. Katz and I. Willner, *J. Electroanal. Chem.*, 1996, **417**, 189.
22 D. G. Davis, *J. Electroanal. Chem.*, 1977, **78**, 383.