A fullerene-modified protein

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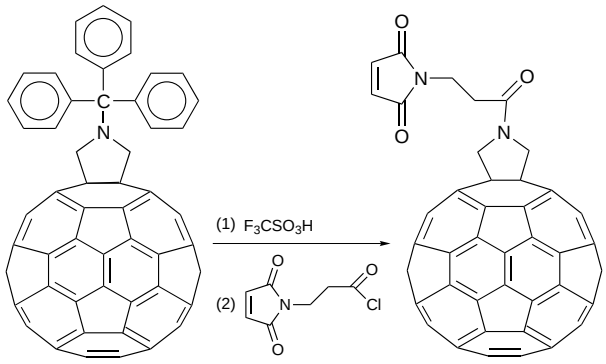
A surface cysteine-containing redox protein (azurin mutant S118C) has been labelled with a C_{60}-based thiol-selective reagent leading to electrochemical interactions between the fullerene and protein redox centre.

In recent years much interest has been drawn to the biological activities of fullerenes, such as cytotoxic activity,[1] selective DNA cleavage[2] and antiviral activity against HIV.[3] However, biological studies have been hindered because of the hydrophobic nature of fullerenes. In an effort to overcome problems associated with their insolubility in aqueous solution, several strategies have been developed such as the formation of complexes with cyclodextrin,[4] liposomes,[5] poly(vinylpyrrolidone)[6] and derivatization with water-solubilising moieties.[7] The C_{60} cage is able to reversibly accept up to six electrons under suitable conditions,[8] so C_{60}, that is covalently attached to biomolecules, can be sensed electrochemically. As C_{60} is insoluble in water little is known about its electrochemical behaviour in aqueous media, although the electrochemistry of films[9] and γ-cyclodextrin inclusion complexes[10] has been studied. The C_{60} radical monoanion has recently[11] been reported to be stable in water and monoanion containing salts have been isolated from aqueous solutions. We report herein the modification of a redox protein (azurin mutant S118C) with buckminsterfullerene and the electrochemical behaviour of the adduct so formed.

Azurin is a small blue copper redox protein which acts as an electron transfer agent in the denitrification chains of a number of bacteria. It has a molecular weight of about 14 000 kDa. As wild type azurin does not contain any surface cysteines (and hence free thiols), a mutant‡ containing just one surface cysteine was created by polymerase chain reaction (PCR) mutagenesis methods[12] and expressed according to published methods.[13] Serine 118 was genetically replaced by a cysteine, this being a conservative mutation to ensure the mutant closely resembled the wild type azurin. The position was chosen to allow labelling close to the redox centre, a copper ion in the case of azurin, in order to investigate the possibility of electrochemical communication between copper and fullerene. The previously described *N*-(triphenylmethyl)-3,4-fulleropyrrolidine[14] was reacted with 3-maleimidopropionyl chloride in the synthesis (Scheme 1) of a thiol selective fullerenomaleimide.§



Scheme 1

This label is not soluble in water and only sparingly soluble in polar organic solvents, but is reactive enough as a suspension to couple to the protein. For the labelling reaction, the fullerenomaleimide (in 100-fold molar excess) was suspended in a 60 μM solution of azurin S118C in 20 mM HEPES buffer at pH 7. The suspension was then stirred for 72 h at 4 °C. The originally blue solution turned green after 24 h. Over the same time period the characteristic electronic absorption band of C_{60} at 330 nm appeared (Fig 1). Unreacted fullerene was removed by size exclusion chromatography (PD-10, Pharmacia). A SDS-PAGE gel (15% polyacrylamide) indicated the presence of a new band at higher mass. The mass increase was estimated to be 5%, consistent with the addition of one fullerene moiety per protein. The reaction appeared to go to completion as no native protein could be detected by SDS-PAGE or by cyclic voltammetry. The cyclic voltammogram of the product shows the presence of a new redox couple at −302 mV assigned to the first electron reduction and reoxidation of C_{60} (Fig 2). The potential lies between that reported[11] for a solution of C_{60} in DMF (−250 mV) and a γ-cyclodextrin inclusion complex of C_{60} in water (−570 mV).[10] The redox couple of the protein copper appears at −42 mV. It is shifted by 41 mV to a more negative potential

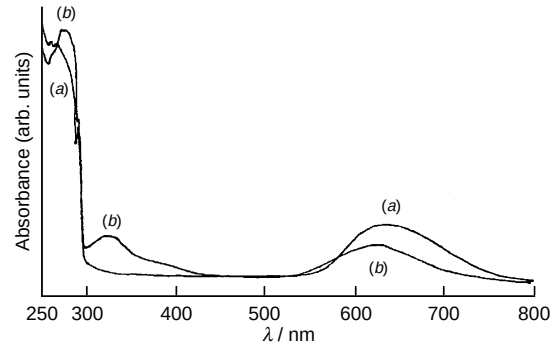


Fig. 1 UV–VIS spectrum (H_2O) of (*a*) azurin S118C and (*b*) fullerene-modifed S118C

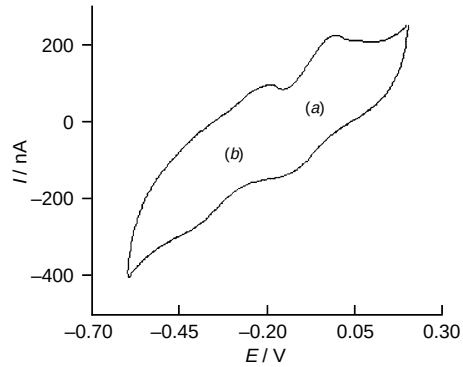


Fig. 2 Cyclic voltammogram at an edge plane graphite electrode of 65 μM fullerene modified S118C in 20 mM HEPES buffer (pH 7.0) containing 5% THF and 5% DMSO at 50 mV s^{-1} and 25 °C. The solutions were purged with argon for 60 min before measurement. (*a*) Copper redox couple Cu^{+2}/Cu^{+1} and (*b*) fullerene redox couple $C_{60}°/C_{60}^{-1}$.

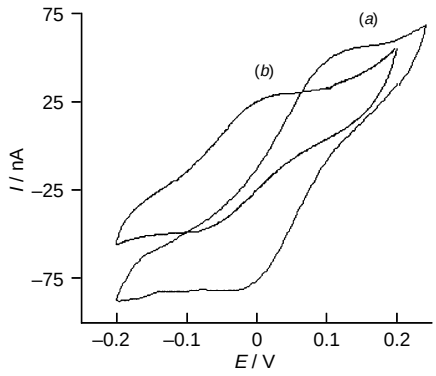


Fig. 3 Cyclic voltammogramm of (*a*) wild type azurin and (*b*) fullerene modified S118C under the same conditions as in Fig 2

compared to the unmodifed protein (−1 mV) under the same experimental conditions (Fig 3). As the fullerene is attached next to histidine 117, which is a copper ligand, modification-induced changes in the redox potential are likely. If the potential range is restricted to that of the azurin copper, a reversible couple is observed with reduction and re-oxidation peak areas of equal magnitude. If scans are continued to more negative potentials (at which the fullerene is reduced) the anodic peak of the Cu couple changes, as does the ratio of anodic to cathodic current. This behaviour suggests communication between copper centre and fullerene. Spectroscopic (UV–VIS) and electrochemical data prove that the fullerene modification did not lead to the denaturation of the protein.

The covalent modification of protein with buckminsterfullerene opens up the possibility of extensive investigations into the aqueous and biological chemistry of these 'fulleryl proteins'.

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Notes and References

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‡ Preparation of S118C: Mutagenesis of azurin *Pseudomonas aeruginosa* was performed by PCR with the primers: 5′GCACCTTCCCGGGC-CAC*TGC*GCGCTGATG-3′ (5′-primer) and 5′-AAACGACGGCCAGT-3′ (3′-primer). The azurin mutant S118C was expressed and stored as the apoform. Dithiothreitol (DTT) was added to the buffers to give a final DTT concentration of 3 mM in order to prevent dimerisation. Prior to labelling the DTT was removed through the use of desalting columns (PD-10, Pharmacia). The protein was reconstituted by addition of an equimolar amount of a 10 mM solution of $Cu(NO_3)_2$.

§ Preparation of *N*-(3-maleimidopropionyl)-3,4-fulleropyrrolidine: *N*-(triphenylmethyl)-3,4-fulleropyrrolidine (30 mmol) (ref. 14) in dry CH_2Cl_2 (10 ml) was treated with trifluoromethanesulfonic acid (100 μl) for 1 h. The precipitate was washed with Et_2O–H_2O and dried. The amine was suspended in dry CH_2Cl_2 (10 ml) and dry pyridine (1 ml). A 30 molar excess of *N*-maleoylpropionyl chloride was added. After stirring for 3 h at room temperature the mixture was purified on silica gel (CH_2Cl_2): δ_H (500 MHz, CS_2–[$^2H_6$]benzene 4 : 1) 6.78 (s 2 H), 5.47 (s 2 H), 5.37 (s 2 H), 3.84 (t 2 H), 3.19 (t 2 H); *m*/*z* (MS-FAB) 913 (M-1, 50%), 720 (C_{60}, 100); λ_{max} (CH_2Cl_2)/nm 232, 260, 332, 406.

1 H. Tokuyama, S. Yamago and E. Nakamura, *J. Am. Chem. Soc.*, 1993, **115**, 7918.
2 A. S. Boutorine, H. Tokuyama, M. Takasugi, H. Isobe, E. Nakamura and C. Hélène, *Angew. Chem., Int. Ed. Engl.*, 1994, **33**, 2462.
3 S. H. Friedman, D. L. DeCaamp, R. P. Sijbesma, G. Srdanov, F. Wudl and G. L. Kenyon, *J. Am. Chem. Soc.*, 1993, **115**, 6506; P. Rajagopalan, F. Wudl, R. F. Schinazi and F. D. Boudinot, *Antimicrob. Agents Chemother.*, 1996, **40**, 2262.
4 T. Andersson, K. Nilsson, M. Sundahl, G.Westman and O. Wennerström, *J. Chem. Soc., Chem. Commun.*, 1992, 604.
5 H. Hungerbühler, D. M. Guldi and K.-D. Asmus, *J. Am. Chem. Soc.*, 1993, **115**, 3386.
6 Y. N. Yamakoshi, T. Yagami, K. Fuhuhara, S. Sueyoshi and N. Miyata, *J. Chem. Soc., Chem. Commun.*, 1994, 517.
7 R. Sijbesma, G. Srdanov, F. Wudl, J. A. Castoro, C. Wilkins, S. H. Friedman, D. L. DeCamp and G. L. Kenyon, *J. Am. Chem. Soc.*,1993, **115**, 6510.
8 Y. Ohsawa and T. Saji, *J. Chem. Soc., Chem. Commun.*, 1992, 781.
9 A. Szücs, A. Loix, J. B. Nagy and L. Lamberts, *J. Electroanal. Chem.*, 1995, **397**, 191; J. J. Davis, H. A. O. Hill, A. Kurz, A. D. Leighton and A. Y. Safronov, *J. Electroanal. Chem.*, 1997, **429**, 7.
10 P. Boulas, W. Kutner, M. T. Jones and K. M. Kadish, *J. Phys. Chem.*, 1994, **98**, 1282.
11 X. Wei, M. Wu, L. Qi and Z. Xu, *J. Chem. Soc., Perkin. Trans. 2*, 1997, 1389.
12 T. J. White, N. Arnheim and H. A. Ehrlich, *Trends Genet.*, 1989, **15**, 185.
13 M. van de Kamp, F. C. Hali, N. Rosato, A Finazzi-Agrò and G. W. Canters, *Biochim. Biophys. Acta*, 1990, **1019**, 283.
14 M. Prato, M. Maggini, C. Giacometti, G. Scorrano, G. Sandonà and G. Farnia, *Tetrahedron*, 1996, **52**, 5221.

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