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Modified cytochrome c/H_2O_2 system: spectroscopic EPR investigation of the biocatalytic behaviour

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Abstract

In recent years there has been growing interest in methods for the degradation of polycyclic aromatic hydrocarbons. Cytochrome *c* (Cyt *c*) systems in the presence of H_2O_2 are able to oxidize various aromatic compounds. In order to investigate ways of improving the performance of Cyt c/H_2O_2 oxidation systems, site-directed mutagenesis, and chemical modifications on the hemoprotein surface with poly(ethylene glycol) and methylation of the active site have been performed. The EPR technique and UV–VIS spectroscopy have been used to identify radical intermediates and heme iron spin states of the chemical modified Cyt *c* (PEG-Cyt-Met) and Cyt *c* mutants.

Keywords: Cytochrome *c*; EPR; Spin trapping; Hemoprotein; Peroxidase activity

1. Introduction

Polycyclic aromatic hydrocarbons are among the most important xenobiotic pollutants. They are considered to be a potential health risk because of their possible carcinogenic and mutagenic activities [1]. Aromatic hydrocarbons have been widespread pollutants from fossil fuel industries during the last century. Because of their chemical stability and hydrophobicity, they are considered as environmentally recalcitrant compounds, especially those with five or more aromatic rings. In fact, their chemical oxidation by presently available techniques requires extreme conditions and can cause further environmental pollution. Biodegradation offers a good solution to dispose of polycyclic aromatic hydrocarbons, but only a few systems can attack such stable aromatic rings.

Hemoprotein systems in the presence of H_2O_2 are able to oxidize various aromatic compounds [2]. Water-miscible organic solvents have been used to reduce the mass transfer limitations for these hydrophobic substrates [3,4]. However, the presence of the organic solvent leads to a decrease of the biocatalytic activity [5]. In addition, hydrogen peroxide, which is the electron acceptor substrate, is able to inactivate the hemoproteins [6,7]. Genetic and chemical modifications of cytochrome *c* (Cyt *c*) have been performed to study ways in which the catalytic performance of such hemoprotein systems can be improved. Site-directed variants of Cyt *c*

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have shown up to a 10-fold increase in catalytic activity compared to the wild-type protein [8]. Chemical modifications on the hemoprotein surface with poly(ethylene glycol) and methylation of the active site have also shown both higher activity and better substrate specificity [9].

In this study EPR spectroscopy, by both spin-trapping of radical intermediates and observation of the heme iron, has been used to determine, at a molecular level, the effect of these chemical and genetic modifications on the different reaction steps.

2. Experimental

2.1. Modified protein preparations

Mutant forms of yeast *iso*-1-Cyt *c* were generated according to Pollock et al. [10]. Recombinant DNA manipulations and construction of the plasmid pBPYC1(wt)/3 were carried out by using the CYC1 gene, which encodes a Thr at the position 102 instead of the wild-type Cys residue. This limits autoreduction of the ferricytochrome and avoids protein dimerization through disulfide bond formation. Bacteria cultures were carried out in a 14-l fermenter containing 10 l of YT medium [11] supplemented with 100 mg/l ampicillin and 0.1% (v/v) glycerol. Isolation and purification of recombinant Cyt *c* were carried out according to Pollock et al. [10]. The final purification was performed on a weak acidic cation exchanger cartridge, Econo Pack CM (BioRad) instead of the MonoQ column used in the original method.

The double chemical modification of Cyt *c* with poly(ethylene glycol) on the protein surface and methyl groups on the free carboxylic groups (poly(ethylene glycol)-Cyt *c* (PEG-Cyt-Met)), was obtained by using activated poly(ethylene glycol) with cyanuric chloride (MW 5000) and a trifluoride–methanol mixture (BF_3–methanol) according to the procedure described previously [9]. This modified preparation includes methylation of the propionate groups of the heme prosthetic group.

2.2. Reagents

Horse heart Cyt *c*, pyrene, fluorene, 2-methyl-2-nitroso-propane (MNP) were purchased from Sigma (St. Louis, MO, USA). 5-Diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO) was purchased from Oxis. Acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, USA).

All the compounds were reagent grade and used without any further purification.

2.3. Procedure

All the solutions were prepared with 0.1 M phosphate buffer at pH 7.4 using distilled deionized water. In order to prevent heavy metal catalyzed secondary radical reactions 200 μM EDTA was present in the buffer. Moreover, the buffer was degassed and stored in a nitrogen atmosphere in order to reduce as far as possible the presence of O_2. This was necessary because O_2 can compete with the spin trap in the reaction with the radical system and, hence, decrease the amount of intermediate compounds.

The MNP solution was prepared freshly before the experiment by carefully dissolving in the dark and in an N_2 atmosphere at 45°C. DEPMPO buffer solutions were stored in the dark and under nitrogen at −80°C.

EPR samples for spin-trapping experiments of the heme-protein/H_2O_2 systems were prepared in such a way that the final concentrations of the reactants were: 0.5 mM of hemoprotein, 18 mM of spin trap and 2.5 mM of H_2O_2. Spectra were recorded 3 min after the addition of the last reactant, which was in all cases the H_2O_2.

Samples of the same systems in the presence of substrate were prepared in the same way, except that the substrate in acetonitrile solution, was added carefully immediately before the last

addition of H_2O_2, in order to obtain a final substrate concentration of 0.5 mM in 25% acetonitrile.

2.4. EPR measurements and simulations

Room temperature EPR spectra were obtained with a Bruker 200D SRC X-band spectrometer. Microwave frequencies were measured with an XL Microwave Model 3120 counter (Jagmar, Krakow, Poland). The spectrometer was interfaced with a PS/2 Technical Instruments Hardware computer and the data acquired using the EPR data system CS-EPR produced by Stelar (Mede, Italy). The instrument parameters were as follows: modulation amplitude 2 G, microwave frequency 9.62 GHz, time constant 200 s, receiver gain 5×10^5, microwave power 63 mW.

Low temperature EPR spectra were recorded on a Bruker EMX spectrometer (Universität Bayreuth, Germany) equipped with an Oxford Instruments ESR 900 cryostat. Recording conditions were: temperature 13 K, modulation amplitude 10 G, microwave frequency 9.47 GHz, microwave power 1 mW. Simulations of EPR spectra were obtained using a computer program written in the author's laboratory and available upon request.

The electronic absorption spectra of the Cyt *c* variants were recorded on a Cary 5 spectrophotometer (University of Florence, Italy).

3. Results and discussion

3.1. Poly(ethylene)glycol-cytochrome c

The chemical modification of horse heart Cyt *c*, giving a methylated PEG-Cyt-Met, changes both catalytic activity and substrate specificity in the reaction with H_2O_2 and organic substrates [8]. The enhanced activity of PEG-Cyt-Met in oxidizing aromatic substrates was attributable to its relatively high hydrophobicity. This would increase the interaction between hydrophobic substrate and active site of the protein [5].

Fig. 1a shows the EPR spectrum of the MNP–(PEG-Cyt-Met) protein radical adduct and the EPR spectrum of the native MNP–Cyt *c* protein radical adduct (Fig. 1b). The latter is paired with the simulation that eventually gave the best fit [12]. The two experimental spectra are quite different and attempts to obtain a good simulation for the spectrum reported in Fig. 1a unfortunately failed. Two possible origins can be proposed for the different lineshape observed in these two cases. The appearance and form of the spectrum for the MNP–(PEG-Cyt-Met) radical is, in general terms, that which is seen on changing from a fast to slow motion domain [13]. The increase in rotational correlation time could be mainly a consequence of the higher molecular weight of the modified enzyme compared to native Cyt *c* (Fig. 1b). In addition, a hydrophobic interaction between the protein radical site and the PEG chains can be envisaged which would have a disrupting influence on the radical site geometry and, hence, modify its EPR spectrum. The latter explanation is favored by viscosity data on the native vs. modified Cyt *c* enzymes having PEG chains. These observations (data not shown) show that the viscosity of the native and modified enzymes has little dependence on molecular weight.

Cyt *c* in the presence of H_2O_2 oxidizes pyrene to form 1,8-pyrenedione [8]. When the substrate pyrene is added to the MNP–(PEG-Cyt-Met) system the spectrum does not change (data not shown) and it is essentially identical to that reported in Fig. 1a. For Cyt *c*, the addition of pyrene gave a spectrum very similar to that recorded for the MNP–(PEG-Cyt-Met)-pyrene system. The fact that a very low molecular weight substrate changes the radical adduct EPR spectrum of Cyt *c* it suggests that there is a hydrophobic interaction between substrate and radical site. This lends further support to the above mentioned proposal that the origin of the spectral differences between the radical adducts

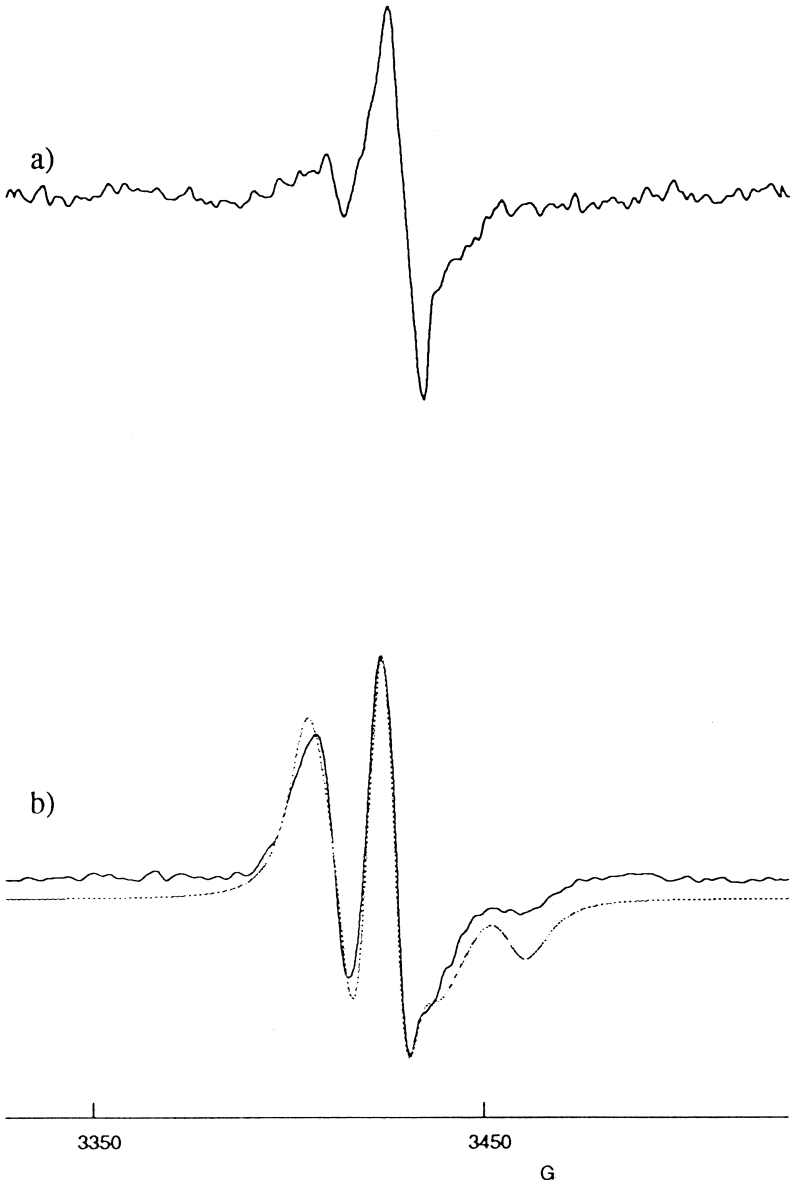

Fig. 1. (a) The EPR spectrum of PEG-Cyt-Met/MNP radical adduct. (b) The EPR spectrum of native Cyt *c*/MNP radical adduct paired with its simulation.

of the native and modified enzymes is a hydrophobic interaction [14].

The use of the DEPMPO spin trap can be useful to characterize any radical oxygen species which may be present. DEPMPO adducts of (PEG-Cyt-Met)–H_2O_2 in the absence (a) and in the presence (b) of pyrene are shown in Fig. 2. The EPR magnetic parameters of the DEPMPO adducts are reported in Table 1. The simulation obtained for the spectrum reported in Fig. 2a has been obtained as a linear combination of a 90% of the DEPMPO-OH and 10% of DEPMPO-OOH adducts. Alkoxyl radicals and peroxyl organic radicals have been detected from Cyt *c* in the presence of organic hydroperoxides [15,16], and has been reported that alkoxyl radi-

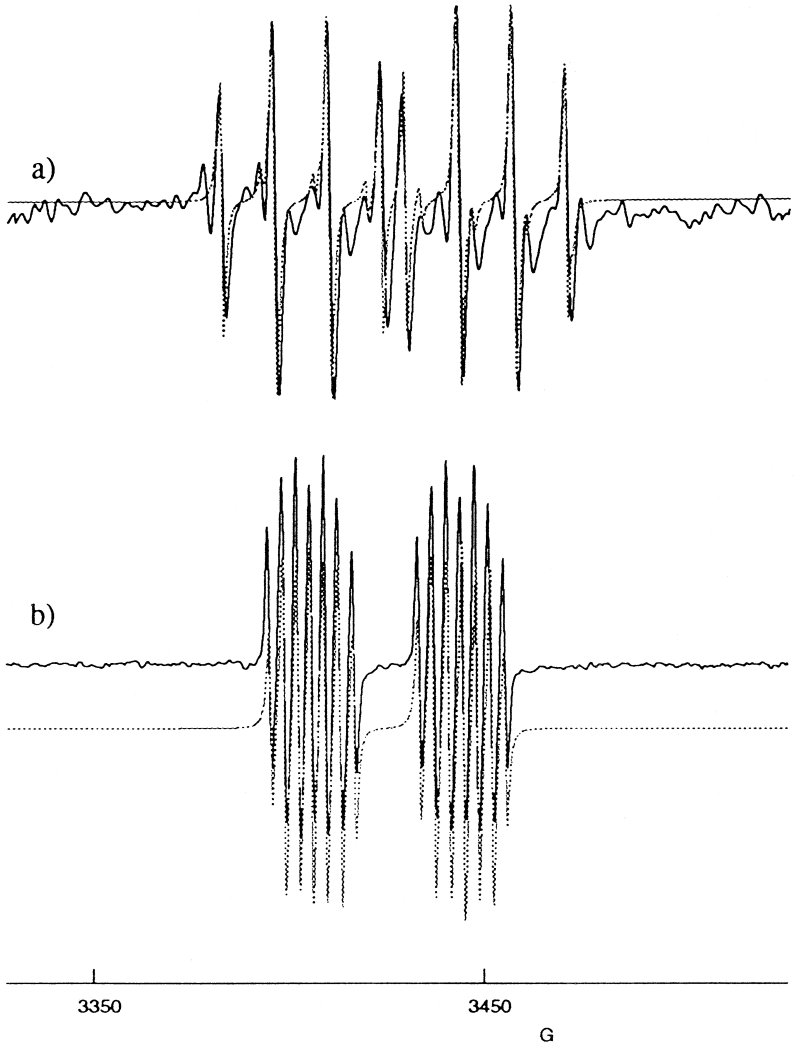

Fig. 2. The EPR spectra of the DEPMPO adducts for the system PEG-Cyt-Met paired with their simulations (a) in the absence of pyrene; (b) in the presence of pyrene.

cals are initially produced and that peroxyl radicals are formed from subsequent reactions of alkoxyl radicals [16]. The EPR spectrum reported of (PEG-Cyt-Met)–H_2O_2 + DEPMPO + pyrene (Fig. 2b) is clearly different from that reported in Fig. 2a. The spectrum reported in Fig. 2b was simulated considering a linear combination of 50% of two species (see Table 1). As the difference between the two systems is only the presence of pyrene, and the DEPMPO spin trap is specific for the oxygen centered radicals, it might be assumed that these two species could be radical intermediates of the oxidation of pyrene to 1,8-pyrenedione, more probably the two conformers [17] of the radical intermediate.

Table 1
The EPR magnetic parameters of DEPMPO adducts of PEG-Cyt-Met/H_2O_2 system in the absence and presence of pyrene[a]

	g	A^P (G)	A^N (G)	A^H (G)
DEPMPO-OH	2.0061	47.3	14.0	13.2
DEPMPO-OOH	2.0061	52.5	13.4	11.9
DEPMPO-ox-pyr	2.0068	49.5	3.62	3.37
DEPMPO-ox-pyr′	2.0069	42.7	3.89	3.37

[a] Estimated errors g: 0.0001, A: 0.1 G.

Using Cyt *c* instead of PEG-Cyt-Met it was not possible to observe the oxidized substrate radical [14]. This was probably due to the high affinity of PEG-Cyt-Met for the substrate. In the latter case the oxidation of the substrate is so efficient that the DEPMPO entraps directly the oxidized species of pyrene that is stable enough to be trapped by the spin trap, giving the spectrum reported in Fig. 2b. These adducts in fact decay rapidly (in about 10 min). Similar interesting results have been obtained using the substrate fluorene that is oxidized by PEG-Cyt-Met but not by Cyt *c*. The spectrum reported in Fig. 3 is clearly a superposition of more than one species. Unfortunately, all attempts to simulate the experimental spectrum using a linear combination of species failed.

Fig. 4 shows the electronic absorption spectra of Cyt *c* and the chemically modified enzyme, PEG-Cyt-Met at room temperature. Prior to use the samples were treated with a minimal quantity (~ 5 μM final concentration) of potassium ferricyanide to ensure that they were fully oxidized. The broadening and 3 nm blue-shift of the Soret band in the modified enzyme compared to native Cyt *c* indicates the presence of some high spin heme in addition to the low spin heme characteristic of native Cyt *c*. The broader Q bands with more intensity at ~ 514 nm and less at ~ 555 nm are also indicative of the presence of high spin and less low spin, respectively, in PEG-Cyt-Met with respect to the native enzyme. The broad band at 620 nm in Cyt-PEG-Met, but absent in the native enzyme, is assigned to the in-plane charge transfer band (CT1) between the porphyrin and heme iron of the high spin heme species. The wavelength of the CT1 for ferric heme species is known to depend on the axial ligand field strength [18]. As in low spin heme species CT1 bands are absent, the relatively low wavelength of the CT1 band in the modified enzyme is suggestive of a hexacoordinate high spin heme in which the sixth ligand is no longer the methionine residue. Methionine ligand, which generates the low spin heme of native Cyt *c*, seems to be replaced by a water molecule which is an H-bond donor [18]. A further clear indication of the different heme spin state in the modified enzyme is the absence of the iron-methionine charge transfer band at 695 nm.

To explore further the altered heme spin state of the modified enzyme compared to native Cyt *c* EPR measurements have been carried out at

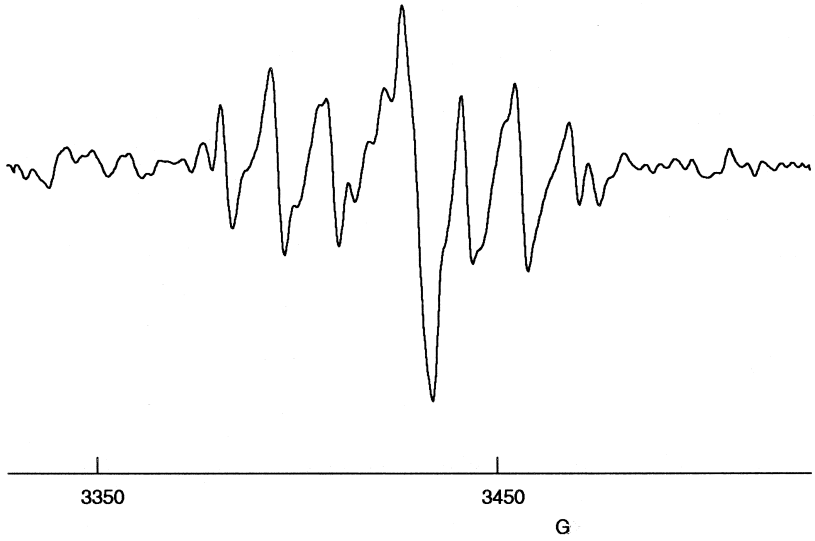

Fig. 3. The EPR spectrum of the DEPMPO adducts for the system PEG-Cyt-Met in the presence of fluorene.

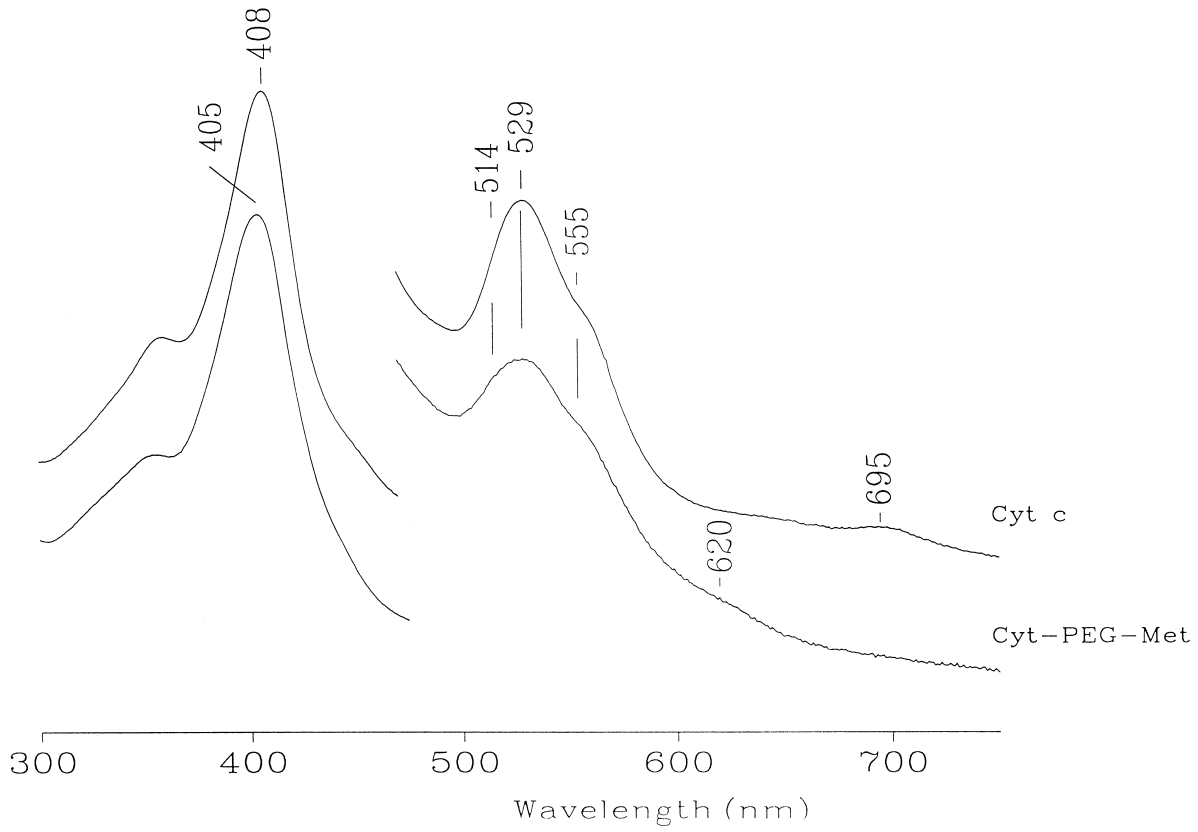

Fig. 4. The UV–VIS absorption spectra of PEG-Cyt-Met and native Cyt *c*.

13 K on frozen solution samples. Fig. 5a shows the typical low spin spectrum of native Cyt *c* ($g = 3.05,\ 2.25,\ 1.25$) [19] which contrasts markedly with that of the modified PEG-Cyt-Met enzyme (Fig. 5b). The latter reveals the presence of both high spin heme ($g_{\perp} = 6.06$, $g_{\parallel} = 2.00$) and low spin heme at $g = 2.76$. The band at $g = 4.3$ results from non-heme rhombic iron often found as an impurity in protein EPR spectra.

The g values of the low spin heme in the modified enzyme clearly indicate that it is not the same low spin species as seen in the native protein and implies a changed axial ligation. The low spin signal with $g = 2.76,\ 2.33$ (g_3 is too weak to be observed) can be categorized as the ''H-type'' described by Blumberg et al. [20], which is characterized by the ligation of an imino nitrogen at the sixth heme iron coordination position. It is difficult to rationalize the origin of the low spin the PEG-Cyt-Met enzyme in these terms, however, as the heme pocket does not have an alternative residue as ligand, it is suggested to result from a weakened Fe-Met bond or iron-bound water molecule, which acquires some hydroxyl character by being a hydrogen-bond donor. If this is the case, the relative contribution of this signal may be expected to change with pH and perhaps also temperature. However, the EPR measurement at low temperature was made only at pH 7.4. In theory, a comparison of the room temperature electronic absorption and low temperature EPR should allow any temperature effects to be determined. The EPR spectrum indicates that the high spin species predominates at low temperature, however, it is not possible to reliably determine the relative contribution of the high and low spin species from the room temperature absorption spectrum.

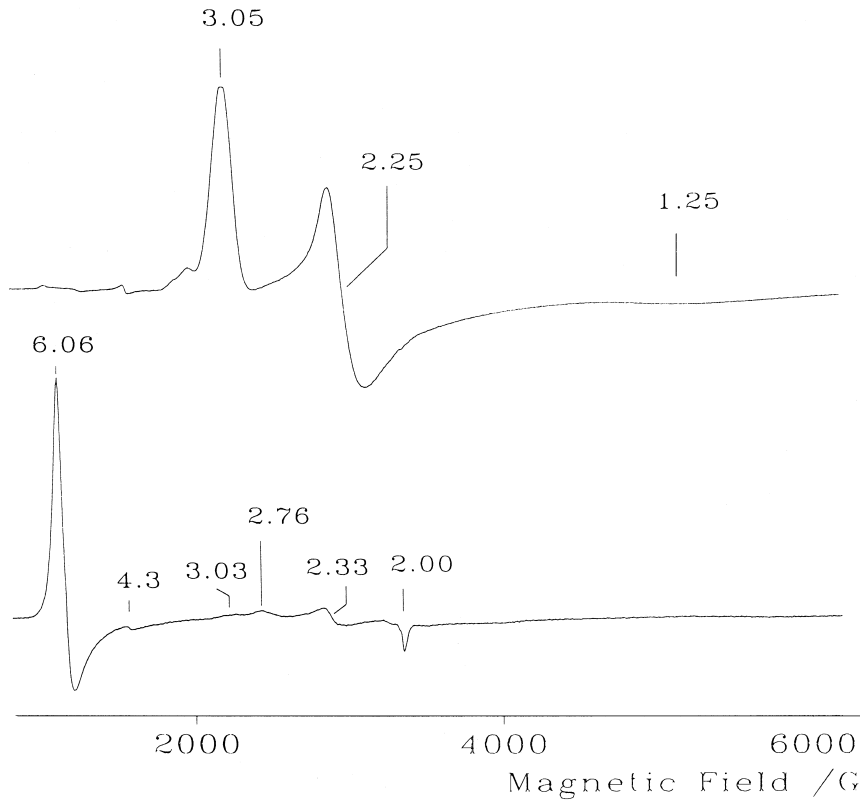

Fig. 5. The EPR spectra at low temperature (13 K) of (a) native Cyt *c*; (b) PEG-Cyt-Met.

Nevertheless, both room temperature electronic absorption and low temperature EPR spectra indicate the presence of a significant proportion of high spin heme in the PEG-Cyt-Met modified enzyme. This implies that in many molecules the iron-methionine bond is ruptured, which may be a contributory factor to the enhanced capacity of the chemically modified enzyme to oxidize organic substrates compared to native Cyt *c* [8]. The relative ease of displacement of the Met-80 sulphur from its position as sixth iron ligand has been noted previously as a consequence of exogenous ligand binding or pH changes [21–23].

It is further suggested that the presence of high spin and low spin heme spin states indicated by both the room temperature electronic absorption and low temperature EPR may result from two allowed dispositions of the Met residue in the distal cavity of the modified enzyme. One close enough to permit strong H-bonding with an iron bound water molecule (generating the low spin state) and one more distant from the iron but still close enough to H-bond with an iron bound water molecule (high spin).

3.2. Phenylalanine 82 variants

Phenylalanine 82 is a phylogenetically conserved residue of Cyt *c*. This residue was modified by site-directed mutagenesis because of its functional role in electron transfer [24], which results from its proximity to the heme prosthetic group. The phenylalanine 82 substitution significantly alters the kinetic behaviour of the protein [8] and, in general, a greater oxidation rate of substrate is accompanied by a decreased stability of the protein towards hydrogen peroxide. In order to investigate the role of the phenylalanine 82 residue in the formation of the

protein radical species, some variants of yeast Cyt *c* were studied in the presence of hydrogen peroxide and the MNP spin trap. The EPR spectra of MNP-protein variant radical adducts

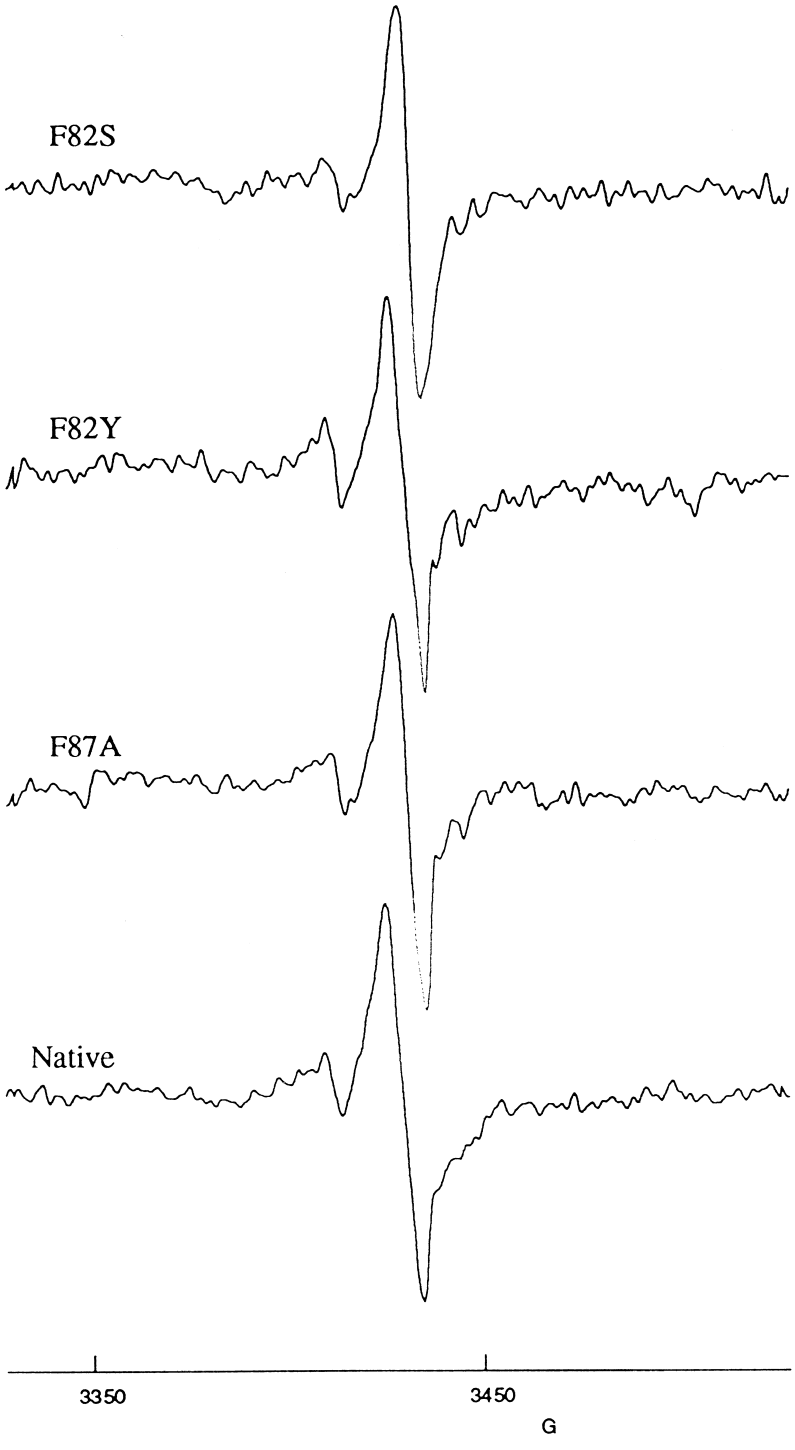

Fig. 6. The EPR spectra of MNP radical adducts of some variants and native Cyt *c*.

in the presence of pyrene appear to be essentially the same as those for the MNP adduct of the native Cyt *c* + pyrene (Fig. 6). This suggests that the phenylalanine residue is not involved in the formation of the protein radical but, more probably, the activity enhancement observed for the phenylalanine 82 variants results from its role as regulator of solvent and hydrogen peroxide access to the heme group [8].

4. Conclusions

In summary, the EPR simulation results showed that DEPMPO-OH and DEPMO-OOH adducts are generated by the modified Cyt c/H_2O_2 system. According electronic absorption spectra and low temperature EPR, the chemically modified preparation, PEG-Cyt-Met, seems to have a disrupted methionine-iron bond, producing a high spin hexacordinate, in which methionine could be no longer the sixth ligand. Finally, the increase of the oxidation activity found in Phe-82 variants of yeast Cyt *c*, could be not attributed to changes in the radical production.

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