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Characterization of the Second Prosthetic Group of the Flavoenzyme NADH–Acceptor Reductase (Component C) of the Methane Mono-oxygenase from *Methylococcus capsulatus* (Bath)

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1. A new two-step purification is described that routinely yields 100mg quantities of component C for biochemical studies. 2. Chemical analyses show component C purified by this procedure to contain 2 g-atoms of iron, 2mol of acid-labile sulphide (S*) and 1 mol of FAD per mol of protein. 3. The Fe–S* core of component C was extruded by treating the protein with *p*-methoxybenzenethiol in hexamethyl phosphoramide/50mM-Tris/HCl buffer, pH8.5 (4:1, v/v), under anaerobic conditions. The spectral properties of the extruded core suggest that component C contains 1mol of [2Fe–2S*(S-Cys)$_4$] centre per mol of protein. 4. E.p.r. spectroscopy confirms the presence of a Fe–S* centre in component C. 5. Component C catalyses the reduction by NADH of ferricyanide, 2,6-dichlorophenol-indophenol or horse heart cytochrome *c*, with specific activities of 50–230 units/mg of protein. 6. The optimum pH for the NADH–acceptor reductase activity is 8.5–9.0, and the apparent K_m values for NADH and NADPH are 0.05mM and 15.5mM respectively. 7. Unlike methane mono-oxygenase activity, NADH–acceptor reductase activity of component C is not inhibited by 8-hydroxyquinoline or by acetylene.

The methane mono-oxygenase complex of *Methylococcus capsulatus* (Bath) catalyses *in vivo* the initial oxygenation of the growth substrate as follows:

$$CH_4 + O_2 + NAD(P)H + H^+ \rightarrow CH_3OH + NAD(P)^+ + H_2O$$

A variety of other alkanes, alkenes, ethers, alicyclic, aromatic and heterocyclic compounds together with NH_3 and CO are substrates that can be used *in vitro* by the enzyme (Colby *et al.*, 1977; Dalton, 1977). In each case the mono-oxygenase catalyses the incorporation of 1 atom of oxygen into the substrate, yielding variously 1- or 2-alcohols, epoxides, cyclic alcohols, phenols, pyridine *N*-oxide, hydroxylamine or CO_2. Enzymes with similar properties occur in two other methane-oxidizing bacteria, *Methylomonas methanica* and *Methylosinus trichosporium* (Stirling *et al.*, 1979).

Methane mono-oxygenase comprises three components (Colby & Dalton, 1978): (i) component A, a large iron-containing protein of mol.wt. about 200000; (ii) component B, a small colourless protein of mol.wt. about 15000; (iii) a metalloflavoprotein, component C. The purification and preliminary characterization of component C were described previously (Colby & Dalton, 1978). It was shown to be a single polypeptide of mol.wt. 44600 with 1 FAD per molecule and non-integral amounts of iron (1.3–1.5 atoms/molecule). The present paper describes an improved two-step purification method for component C that yields large quantities of protein with integral values for FAD, iron and acid-labile sulphide (S*) contents. The results of core-extrusion and e.p.r.-spectroscopy studies are also described, and these have led to the identification of the second non-flavin prosthetic group of component C.

Materials and Methods

Materials

Hexamethyl phosphoramide, horse heart cytochrome *c*, Sephadex G-100 and NADPH were obtained from Sigma Chemical Co., Poole, Dorset, U.K. NADH was from Boehringer Corp., Lewes, Sussex, U.K. BDH Chemicals, Poole, Dorset, U.K., supplied $K_3Fe(CN)_6$, and *p*-methoxybenzenethiol was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K.

Methods

Growth of *M. capsulatus* (Bath) and preparation of soluble extracts was done as described previously (Colby & Dalton, 1978). Resolution of methane mono-oxygenase activity into fractions A, B and C was achieved by ion-exchange chromatography on a column of DEAE-cellulose (Colby & Dalton, 1978); fraction C bound to the column packing and was subsequently eluted with 0.5M-NaCl.

Purification of component C. All procedures were done at 4°C.

Step 1. Fraction C (about 70ml, 420mg of protein) in 20mM-Tris/HCl buffer, pH7, containing 5mM-sodium thioglycollate and 0.5M-NaCl, was pumped at 60ml/h on to a column (1.5cm × 11cm) of 5'-AMP–Sepharose 4B (Pharmacia Fine Chemicals, London W5 5SS, U.K.) which had been equilibrated with 20mM-Tris/HCl buffer, pH7. Component C bound to the column packing and, after washing with the equilibrating buffer, was eluted with the same buffer containing 1mM-NADH. Component C was eluted from the column as a purple band, but its yellow–brown colour was restored on exposure to the air.

Step 2. Two batches of component C from step 1 were combined and concentrated to 2ml by ultrafiltration through an Amicon PM30 membrane (Amicon, High Wycombe, Bucks., U.K.). The dark-brown concentrate was placed on a column (2.5cm × 100cm) of Sephadex G-100 equilibrated with 20mM-Tris/HCl buffer, pH7, containing 5mM-sodium thioglycollate, and eluted with the same buffer at 50ml/h. The eluate was monitored for component C by measuring its absorbance at 465nm. The fractions absorbing at this wavelength were combined, concentrated by ultrafiltration as above, and then dialysed for 20h against 1000vol. of 5mM-Tris/HCl buffer, pH7, with two changes of dialysing buffer. Samples of component C for core-extrusion studies were dialysed instead against 50mM-Tris/HCl buffer, pH8.5.

Enzyme assays. All assays were done at 45°C, which is the optimum growth temperature for *M. capsulatus* (Bath). Methane mono-oxygenase activity was determined by following the oxidation of ethene to epoxyethane as described by Colby & Dalton (1978). NAD(P)H–acceptor reductase activities of component C were measured by following the reduction of the acceptor spectrophotometrically at 410nm for $K_3Fe(CN)_6$, 600nm for 2,6-dichlorophenol-indophenol and 550nm for cytochrome *c*. Reaction mixtures (1ml final volume) contained: 50μmol of sodium phosphate buffer, pH7; electron acceptor (0.9μmol of $K_3Fe(CN)_6$, 0.09μmol of 2,6-dichlorophenol-indophenol or 0.05μmol of cytochrome *c*); 1μmol of NADH or NADPH. Reactions were started by adding component C (1–5μg of protein).

Polyacrylamide-gel electrophoresis. The purity of component C preparations was assessed electrophoretically as described previously (Colby & Dalton, 1978). Component C activity was detected as a purple band on non-denaturing cylindrical gels by incubating them in the dark at 45°C in 10ml of 50mM-sodium phosphate buffer, pH7, containing NADH (0.5mM) and Nitro Blue Tetrazolium (10mg).

Colorimetric analyses. (*a*) Protein was assayed with the Folin–Ciocalteu reagent (Kennedy & Fewson, 1968), with dried crystalline bovine plasma albumin as standard.

(*b*) Iron was assayed as described by Colby & Dalton (1978).

(*c*) Acid-labile sulphide was assayed by the method of Brumby *et al.* (1965). In calculating the results an ε_{mM} of 30.5 litre · mmol^{-1} · cm^{-1} was assumed for the coloured product (Chen & Mortenson, 1977).

(*d*) FAD was assayed by diluting purified component C appropriately in 20mM-sodium phosphate buffer, pH7, containing 1% (w/v) sodium dodecyl sulphate. The solution was placed in a boiling-water bath in the dark for 10min and then, after cooling, its absorbance at 450nm measured. FAD concentrations were calculated by using an ε_{mM} value of 11.3 litre · mmol^{-1} · cm^{-1} (Beinert, 1960).

Extrusion of the Fe-S core of component C*

This was done at 25°C by established methods (Hill *et al.*, 1977; Gillum *et al.*, 1977). The thiol reagent *p*-methoxybenzenethiol rather than benzenethiol was used to take advantage of the longer-wavelength absorption maxima of its [2Fe–2S*] and [4Fe–4S*] complexes (Hill *et al.*, 1977), thus minimizing the interference by the flavin prosthetic group. Reactions were done in 1.5ml quartz cuvettes capped with Suba-Seal stoppers and containing 0.8ml of hexamethyl phosphoramide and 0.2ml of component C solution (0–0.05mM) in 50mM-Tris/HCl buffer, pH8.5. The aqueous component of the solvent was buffered to pH8.5 to prevent spontaneous conversions of [2Fe–2S*] into [4Fe–4S*] (Gillum *et al.*, 1977). Before the addition of the thiol (5μl), cuvettes were evacuated several times on a glass manifold vacuum line, filling the gas space with argon at each cycle. The cuvettes were kept in the dark during the course of the reaction, except when spectra were being recorded by using a Pye–Unicam 1800 spectrophotometer.

E.p.r. spectroscopy

E.p.r. measurements were made on a Varian E9 spectrometer by Dr. D. J. Lowe of the ARC Unit of Nitrogen Fixation, University of Sussex, Brighton, U.K. The techniques used were those described by Lowe *et al.* (1972) and Bray *et al.* (1978).

Experimental and Results

Purification of component C

Affinity chromatography of fraction C on 5'-AMP–Sepharose 4B results in a 48-fold purification over crude soluble extract with 45% yield. The resulting material shows only faint contaminating protein bands on polyacrylamide-gel electrophoresis. These can be removed by a subsequent Sephadex G-100 chromatography step, although with the loss of 22% of the component C activity. At this stage component

Table 1. *Purification of component C*

The methane mono-oxygenase activity of component C was determined in the presence of fraction A (4mg of protein) and fraction B (2.1mg of protein) as described in the Materials and Methods section. The enzyme units are μmol of epoxyethane formed/min.

Stage	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg of protein)	Yield (%)	Purification factor
Crude soluble extract	240.0	1770	2130	0.12	100	1
Fraction C	128.0	900	1620	1.80	76	15
AMP–Sepharose eluate	16.6	165	960	5.80	45	48
Sephadex G-100 eluate	48.0	124	745	6.00	35	50

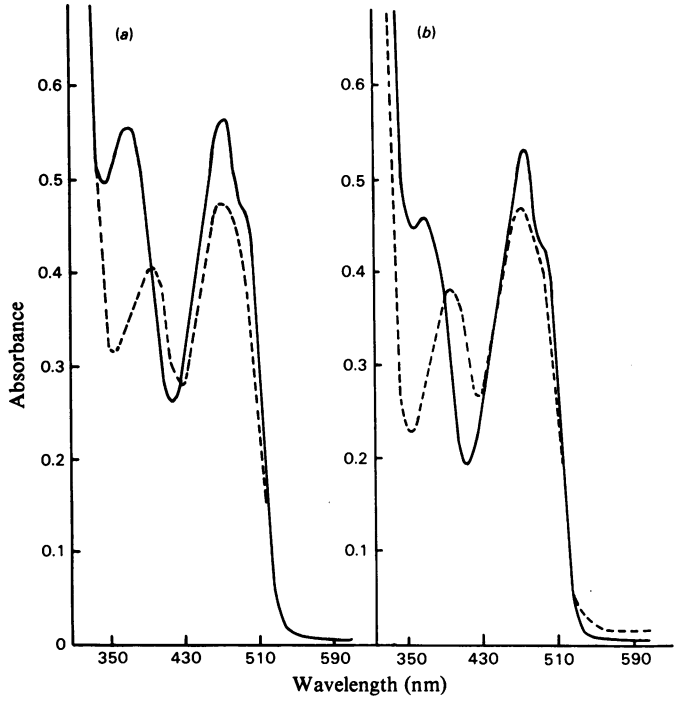


Fig. 1. *Effect of solvent on the absorption spectra of component C* (0.043mM; *b*) *and of authentic FAD* (0.043mM; *a*)
Details are given in the Experimental and Results section. ----, Spectra recorded in 50mM-sodium phosphate buffer, pH7, containing 1% (w/v) sodium dodecyl sulphate. ——, Spectra recorded in hexamethyl phosphoramide/50mM-Tris/HCl buffer, pH8.5 (4:1, v/v).

C is electrophoretically homogeneous. Moreover, the single protein band on non-denaturing cylindrical gels has the same relative mobility as the band of NADH–Nitro Blue Tetrazolium reductase activity detected on the gel as described in the Materials and Methods section.

Table 1 summarizes the results of a typical purification run. This procedure is a considerable improvement on that published previously (Colby & Dalton, 1978), giving three times the yield and involving only half the number of steps.

Flavin, iron and acid-labile sulphide contents of component C

Colorimetric analyses, done as described in the Materials and Methods section, showed purified component C to contain 1.00mol of FAD, 1.92g-atoms of iron and 2.06mol of acid-labile sulphide per mol of protein. These are average values from three preparations, calculated assuming a mol.wt. of 44600 (Colby & Dalton, 1978).

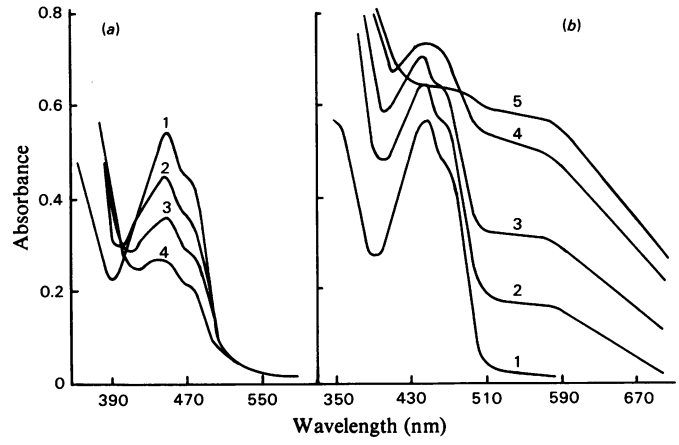


Fig. 2. *Absorption spectra of FAD* (0.043 mM) *and of component C* (0.043 mM) *in hexamethyl phosphoramide/50 mM-Tris/HCl buffer, pH* 8.5 (4:1, *v/v*) *at various times after adding* 40 μmol *of p-methoxybenzenethiol*

Reaction mixtures (1 ml) were incubated in capped 1 ml quartz cuvettes under argon at 25°C throughout the course of the reactions. (*a*) Curve 1, spectrum recorded before adding thiol; curve 2, 30 min after adding thiol; curve 3, 60 min after adding thiol; curve 4, 120 min after adding thiol. (*b*) Curve 1, spectrum recorded before adding thiol; curve 2, 15 min after adding thiol; curve 3, 30 min after adding thiol; curve 4, 60 min after adding thiol; curve 5, 120 min after adding thiol.

Extrusion of an Fe–S core from component C*

Boiling component C (0.043 mM) with 1% (w/v) sodium dodecyl sulphate in aqueous buffer results in the release of FAD and a change in its absorption spectrum to that typical of FAD with maxima at 450 nm and 375 nm (Fig. 1, broken lines); native component C has a single maximum in the visible region at 465 nm with a shoulder at 395 nm (Colby & Dalton, 1978). Incubation of component C (0.043 μmol) at 25°C with 1 ml of hexamethyl phosphoramide/50 mM-Tris/HCl buffer, pH 8.5 (4:1, v/v), however, results in a different absorption spectrum with maxima at 450 nm and 345 nm and a pronounced shoulder at 475 nm (Fig. 1*a*, solid line). Identical changes in the spectrum of authentic FAD (0.043 μmol) are noted on incubation at 25°C in 1 ml of the same solvent mixture (Fig. 1*b*, solid line). These results indicate that incubation of component C in this aprotic aqueous mixture brings about the release of its flavin prosthetic group.

Both the FAD and the component C solutions were then made anaerobic as described in the Materials and Methods section, and 5 μl (40 μmol) of *p*-methoxybenzenethiol was added to each. The absorption spectrum of the FAD solution showed a slow decrease in the absorption peak at 450 nm until a stable spectrum was obtained after 120 min (Fig. 2*a*). The changes in the spectrum of the component C solution were completely different, with steadily increasing absorption at longer wavelengths. Again a stable spectrum was observed after 120 min, and at this stage the solution was dark brown with a broad absorption maximum at about 450–590 nm (Fig. 2*b*). The difference spectrum (component C minus FAD), representing the spectrum of the Fe-S* core complex extruded from component C, showed a peak at 510–550 nm with a maximum absorption of 0.54 (Fig. 3). The shape and position of this absorption peak is similar to that observed with other [2Fe–2S*] proteins, such as spinach ferredoxin (Hill *et al.*, 1977) and to the spectra of synthetic $[2Fe–2S^*(SR)_4]^{2-}$ complexes (Mayerle *et al.*, 1975); they differ from the absorption characteristics of extruded $[4Fe–4S^*(SR)_4]^{2-}$ complexes (Hill *et al.*, 1977) which have an absorption peak at 470 nm. The core extrusion was repeated with different concentrations of component C, and the A_{510} value obtained after 2 h incubation with the thiol reagent measured. A linear relationship was obtained and, assuming an ε_{mM} value for the extruded $[2Fe–2S^*(SR)_4]^{2-}$ of 11.2 (Mayerle *et al.*, 1975), the data indicate that component C contains 0.96 [2Fe–2S*] centres per mol of protein and are therefore consistent with the analytical results presented above.

E.p.r. spectroscopy

Native component C gave no e.p.r.-detectable species (Fig. 4*a*). Reduction of the flavoprotein with NADH (Fig. 4*b*) gave rise to a free-radical signal at $g = 2.002$ (due to the flavin semiquinone) and the signal of the reduced Fe–S* centre with rhombic symmetry and g values of 2.047, 1.960 and 1.864 ($g_{av.} = 1.957$). On further reduction of the protein with solid sodium dithionite (Fig. 4*c*) the signal due

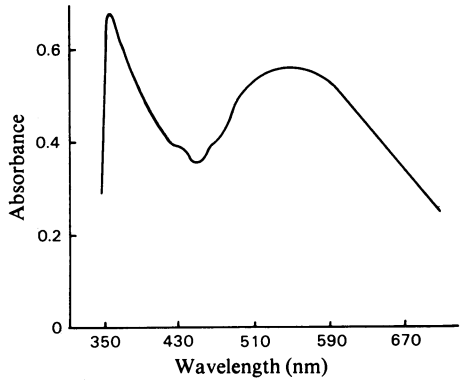


Fig. 3. *Absorption spectrum of Fe–S* core extruded from component C*

Difference spectrum (Fig. 2*b* curve 5 minus Fig. 2*a* curve 4) between thiol-treated component C and thiol-treated FAD recorded 120min after the addition of 40μmol of *p*-methoxybenzenethiol.

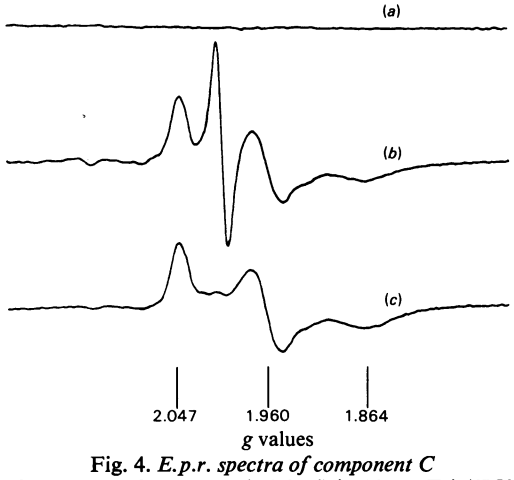


Fig. 4. *E.p.r. spectra of component C*

Component C (0.07μmol; 0.3ml) in 50mM-Tris/HCl buffer, pH7, was made anaerobic by repeated evacuation and flushing with argon (*a*). NADH (5μmol) was added anaerobically to the sample and the spectrum recorded after incubation for 1min at room temperature (*b*). The fully reduced spectrum (*c*) was obtained by adding a few crystals of solid sodium dithionite to sample (*b*) and leaving it at room temperature for 1min. The spectra were recorded at 19K at a microwave frequency of 9.232GHz and modulation of 1.0mT. The microwave powers were: (*a*) 10mW; (*b*) 1mW; (*c*) 1mW.

to the flavin semiquinone was abolished, but there was no significant change in the intensity of the signal due to the Fe–S* centre.

Catalytic activities of component C

Purified component C is active in the methane mono-oxygenase assay when supplemented with fractions A and B. In addition, component C alone catalyses the reduction by NADH of $K_3Fe(CN)_6$ (specific activity 230 units/mg of protein), 2,6-dichlorophenol-indophenol (50 units/mg of protein) and horse heart cytochrome *c* (180 units/mg of protein). (The units of activity are μmol of acceptor reduced/min.) In each case activity is linearly related to component C concentration and is destroyed by heating the protein at 70°C for 10min. Activities with NADPH (1mM) are only 2–3% of the activities with NADH (1mM). The rate of NADH (1mM) oxidation with oxygen as acceptor (NADH oxidase activity) was 0.09 unit/mg, and there was no detectable NADH–oxidized glutathione reductase activity.

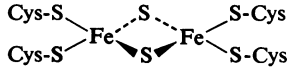
The properties of the NADH–acceptor reductase activity were investigated with 2,6-dichlorophenol-indophenol as acceptor. The optimum pH for this activity was 8.5–9.0, compared with 6.5–7.0 for methane mono-oxygenase activity (Colby & Dalton, 1976). The apparent K_m values for NADH and NADPH at pH7, estimated by the direct-linear-plot method of Eisenthal & Cornish-Bowden (1974), were 50μM and 15.5mM respectively: $V_{max.}$ values were 76 units/mg of protein for NADH and 7 units/mg for NADPH. Like methane mono-oxygenase activity (Stirling & Dalton, 1977), NADH–acceptor reductase activity was unaffected by the chelating agents EDTA (1mM), KCN (1mM), $\alpha\alpha$-bipyridine (1mM) and neocuproine (1mM). Unlike methane mono-oxygenase activity, however, this activity of component C was not inhibited by 8-hydroxyquinoline (1mM) nor by acetylene (0.5mM).

Discussion

Previously the observation that the FAD of component C could be reduced by NADH was taken to suggest that C is the reductase component of the methane mono-oxygenase complex (Colby & Dalton, 1978). Confirmatory evidence for this hypothesis has come from (1) the ability of component C to bind to 5′-AMP–Sepharose 4B and to be eluted with NADH, suggesting that it has a binding site for NADH, and (ii) the ability of component C to catalyse the transfer of electrons from NADH to a number of acceptors. The 300-fold difference in the apparent K_m values for NADH and NADPH provides good evidence that the natural electron donor is NADH rather than NADPH. The two known inhibitors of methane mono-oxygenase activity *in vitro* (discounting other acetylenic compounds), 8-hydroxyquinoline and acetylene (Stirling & Dalton, 1977), do not inhibit the NADH–acceptor reductase activity of component C. This suggests that these inhibitors act on one of the other components of the complex. However, the reductase activity is not a reliable indicator of the activity of component C in the methane mono-

oxygenase assay; preparations of component C have been obtained that have lost their activity in the methane mono-oxygenase assay although retaining full reductase activity. Inhibition by these two inhibitors of the ability of component C to donate electrons to the other components of the complex cannot be ruled out at this stage.

Earlier work showed component C to contain tightly bound iron, although not in integral amounts (Colby & Dalton, 1978). The new purification procedure described above yields preparations of component C that contain very nearly 2mol of iron and 2mol of acid-labile sulphide per mol of protein. [2Fe–2S] proteins, such as spinach ferredoxin, adrenodoxin and putidaredoxin, are thought to have an active site consisting of the illustrated structure



which in the oxidized protein contains two tetrahedrally co-ordinated high-spin anti-ferromagnetically coupled Fe(III) ions (Gillum *et al.*, 1976). $[2Fe–2S^*(S\text{-}Cys)_4]$ and $[4Fe–4S^*(S\text{-}Cys)_4]$ proteins have been shown to undergo a ligand-substitution reaction when incubated with excess thiol (e.g. *p*-methoxybenzenethiol) in aprotic aqueous solvent mixtures (Que *et al.*, 1975; Gillum *et al.*, 1977; Erbes *et al.*, 1975; Howard *et al.*, 1976; Hill *et al.*, 1977). In the case of [2Fe–2S] proteins this reaction can be represented as:

$$\text{Holoprotein} + \text{RSH} \rightarrow [2Fe–2S^*(SR)_4]^{2-} + \text{apoprotein}$$

The Fe–S core is extruded as the thiol analogue $[2Fe–2S^*(SR)_4]^{2-}$, which has a characteristic absorption spectrum that allows its identification, and, from the magnitude of its absorption, the amount of extruded core can be calculated. Treatment of component C with *p*-methoxybenzenethiol in hexamethyl phosphoramide/aqueous buffer (4:1, v/v) led to the formation of a coloured complex with the absorption characteristics of a $[2Fe–2S^*(SR)_4]^{2-}$ cluster similar to those found with other [2Fe–2S] proteins of the plant ferredoxin type.

E.p.r. spectroscopy confirmed the presence of an iron–sulphide centre in component C. The iron signal obtained was typical of a reduced iron–sulphide protein of the $[2Fe–2S^*(S\text{-}Cys)_4]$ class (Orme-Johnson, 1973). In addition, further evidence was obtained for flavin semiquinone formation on reduction of component C with NADH (cf. Colby & Dalton, 1978).

Component C shares some properties with adrenodoxin reductase, NADH–rubredoxin reductase, putidaredoxin reductase and NADH–cytochrome b_5 reductase, each of which is the reductase component of a NAD(P)H-requiring mono-oxygenase system (see Colby & Dalton, 1978). It differs from these enzymes in having a [2Fe–2S*] centre of the plant ferredoxin type in addition to FAD. Three of these other flavoprotein reductases interact with non-haem-iron electron-carrier proteins, i.e. putidaredoxin $[2Fe–2S^*(S\text{-}Cys)_4]$, adrenodoxin $[2Fe–2S^*(S\text{-}Cys)_4]$ and rubredoxin $[Fe(S\text{-}Cys)_4]$. It is tempting to speculate that a single polypeptide, component C, having both flavin and Fe–S* prosthetic groups, plays the same role in the methane mono-oxygenase complex as the flavoprotein reductase/Fe–S* protein combinations found in these other mono-oxygenase systems.

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