An investigation of the iron–sulphur proteins of benzene dioxygenase from *Pseudomonas putida* by electron-spin-resonance spectroscopy

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Benzene dioxygenase from *Pseudomonas putida* comprises three components, namely a flavoprotein (NADH:ferredoxin oxidoreductase; M_r 81000), an intermediate electron-transfer protein, or ferredoxin (M_r 12000) with a [2Fe-2S] cluster, and a terminal dioxygenase containing two [2Fe-2S] iron–sulphur clusters (M_r 215000), which requires two additional Fe^{2+} atoms/molecule for oxygenase activity. The ferredoxin and the dioxygenase give e.s.r. signals in the reduced state with rhombic symmetry and average g values of 1.92 and 1.896 respectively. The mid-point redox potentials were determined by e.s.r. titration at pH 7.0 to be -155 mV and -112 mV respectively. The signal from the dioxygenase shows pronounced g anisotropy and most closely resembles those of 4-methoxybenzoate mono-oxygenase from *Pseudomonas putida* and the [2Fe-2S] 'Rieske' proteins of the quinone–cytochrome c region of electron-transport chains of respiration and photosynthesis.

A strain of *Pseudomonas putida* grown with benzene as the sole source of carbon has been shown to contain a soluble enzyme system that converts benzene into *cis*-benzene glycol (*cis*-1,2-dihydroxycyclohexa-3,5-diene) in the presence of NADH, O_2 and Fe^{2+} (Axcell & Geary, 1975). The system consists of three proteins: a flavoprotein, an intermediate electron-transfer iron–sulphur protein (referred to below as the ferredoxin), and a terminal dioxygenase, which is also an iron–sulphur protein and contains four atoms each of iron and inorganic sulphur per molecule (Crutcher & Geary, 1979) (Scheme 1). It has been demonstrated by cluster-extrusion experiments (Crutcher & Geary, 1979) and by Mössbauer spectroscopy (Geary & Dickson, 1981) that these are arranged in two [2Fe-2S] clusters. In addition to benzene dioxygenase, it has been reported that four other oxygen-

ase enzymes contain iron–sulphur clusters (Bill *et al.*, 1981; Yamaguchi & Fujisawa, 1980; Sauber *et al.*, 1977; Subramanian *et al.*, 1979).

In the absence of added Fe^{2+} , pure preparations of the dioxygenase exhibit low oxygenase activity (less than 10% of a control activated with Fe^{2+}) in combination with the other two proteins of the electron-transport chain, although reduction of the iron–sulphur clusters may be demonstrated anaerobically by using NADH and the complete enzyme system (Crutcher & Geary, 1979). The addition of Fe^{2+} to reaction mixtures increases the oxygenase activity. Since the electron-transport chain from NADH to the dioxygenase iron–sulphur clusters functions in the absence of added Fe^{2+} , it seems likely that an additional iron-binding site is present on the dioxygenase that acts as the oxygen-binding and activation centre in a way similar to the mononuclear non-haem iron centre described by Twilfer *et al.* (1981).

E.s.r. experiments have now been performed on the ferredoxin and on the dioxygenase to determine the characteristics of their iron–sulphur centres and their redox potentials. The number of iron atoms, in addition to cluster iron, bound per molecule of active dioxygenase has also been determined with a view to defining the nature of the active site. The results of these investigations are reported in the present paper.

Scheme 1. Organization of proteins in benzene dioxygenase. Fp, flavoprotein (component A2); Fd, intermediate electron-transfer protein (ferredoxin, component B); Oxygenase, terminal dioxygenase (component A1) [see Crutcher & Geary (1979)].

Experimental

Preparation of benzene dioxygenase proteins

Cells of *Pseudomonas putida* were grown with benzene as the sole source of carbon (Axcell & Geary, 1973). The two iron-sulphur proteins were purified by the method of Crutcher & Geary (1979), except that Sephacryl S-200 (Pharmacia, Uppsala, Sweden) was substituted for Bio-Gel A-0.5m to improve resolution of chromatographic steps. The flavoprotein obtained by this procedure was further treated with FAD and purified in accordance with the method given in Geary & Dickson (1981). Proteins were stored at -22°C in small batches until required. The protein concentration of samples was determined by using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Watford, Herts., U.K.) or by the method of Lowry *et al.* (1951). Calibration curves were prepared with dry samples of bovine serum albumin and catalase and also with desalted freeze-dried preparations of the proteins under study. Potassium phosphate buffer (25mM, pH 7.3) was used throughout except as specified in the text.

Assay of benzene dioxygenase activity

Oxygen consumption was measured at 30°C with a Rank oxygen electrode (Rank Bros., Bottisham, Cambs., U.K.). Reaction mixtures contained, in a total volume of 3ml of buffer, suitable amounts of the three protein components, $1\mu\text{mol}$ of NADH and $1.8\mu\text{mol}$ of FeSO_4 (when required; in excess of the amount required for maximum activation under these conditions). After the endogenous rate had been recorded, the reaction was initiated by the addition of $0.56\mu\text{mol}$ of benzene. The concentrations of NADH and the flavoprotein were chosen to be saturating. Under the conditions specified the reaction rate was found to be linear with respect to the concentration of both the oxygenase and the ferredoxin. All rates are corrected for endogenous respiration.

E.s.r. measurements

E.s.r. spectra were recorded with a Varian E4 spectrometer (Varian, Zug, Switzerland) equipped with an Oxford Instruments liquid-He cryostat (Oxford Instruments, Oxford, U.K.). Measurements of spin concentration were by double integration with correction for baseline, with Cu-EDTA as standard. Redox titrations were performed by the method of Dutton (1978). The proteins were titrated in 100mM-Mops (4-morpholinepropanesulphonic acid)/NaOH buffer, pH 7.0, in the presence of the following mediators: Methylene Blue, Indigotetrasulphonate, Indigodisulphonate, anthraquinone-2-sulphonate, anthraquinone-2,7-disulphonate, Phenosafranin and

Benzyl Viologen. The potential was adjusted with 200mM- $\text{K}_3\text{Fe}(\text{CN})_6$ and 100mM- $\text{Na}_2\text{S}_2\text{O}_4$, and measured with platinum and calomel electrodes. After equilibration for 2min, a sample was withdrawn anaerobically with a syringe and frozen for e.s.r. measurements.

Re-activation of benzene dioxygenase by Fe^{2+}

A modification of the method described by Bill *et al.* (1981) was used. We have previously reported the incorporation of iron into the dioxygenase protein by incubation at pH 6.6 in succinate/NaOH buffer with a 2–5-fold molar excess of FeSO_4 , followed by removal of the excess by gel filtration. Protein thus activated contained 0.8mol of Fe/mol in addition to cluster Fe, and had 60% of the activity of protein activated by the addition of Fe^{2+} (saturating) to the reaction mixture (Geary & Dickson, 1981). This study has now been extended by employing the addition of iron under reducing conditions in the presence of the thiol compounds dithiothreitol and 2-mercaptoethanol.

The effect of the compounds on re-activation of the dioxygenase was first tested individually and in combination. The dioxygenase ($100\mu\text{l}$; 23.5nmol) in 0.1M-Tris/HCl buffer, pH 7.5, was incubated with 4mM- FeSO_4 supplemented with either 16mM-dithiothreitol or 100mM-mercaptoethanol or with a combination of both in a total volume of $200\mu\text{l}$ of 0.1M-Tris/HCl buffer, pH 7.5, for 60min at 4°C under N_2 . At the end of this time $30\mu\text{l}$ samples were withdrawn in duplicate and assayed for oxygenase activity. Results were compared with those from a control activated by the addition of Fe^{2+} directly to the reaction mixture.

In order to determine the number of iron atoms bound per molecule of protein, the protein was similarly activated in the presence of benzene and $\text{Na}_2\text{S}_2\text{O}_4$. The reaction mixture contained 50.5mg (235nmol) of the dioxygenase in 0.1M-Tris/HCl buffer, pH 7.5, $100\mu\text{l}$ of 160mM-dithiothreitol and $100\mu\text{l}$ of 1M-mercaptoethanol in the same buffer, $100\mu\text{l}$ of 40mM- $\text{Na}_2\text{S}_2\text{O}_4$ in 1.0M-Tris/HCl buffer, pH 8.0, $2\mu\text{l}$ of benzene and $100\mu\text{l}$ of 40mM- FeSO_4 . It was incubated for 20min at 4°C under N_2 , after which excess unbound iron and reductants were separated on a column ($45\text{cm} \times 3\text{cm}^2$) of Sephadex G-25 (superfine grade; Pharmacia, Uppsala, Sweden) eluted with 0.1M-Tris/HCl buffer, pH 7.5, containing 5mM-benzene. Fractions (1ml) were collected, and the contents of the centre tubes from the protein peak were pooled and concentrated to approx. 1ml by using an Amicon ultrafiltration cell with a PM-10 membrane (Amicon, High Wycombe, Bucks., U.K.). The concentrate ($45\mu\text{l}$) was assayed immediately for activity towards benzene and compared with a control activated by Fe^{2+} added directly to the reaction mixture. Protein con-

centrations before and after column chromatography were determined. Total iron content before and after activation was determined by the method of Zabinski *et al.* (1972) as modified by Crutcher & Geary (1979). The experiment was repeated with a second sample of the dioxygenase.

Ultracentrifuge measurements

Ultracentrifuge measurements were made with a Beckman model E analytical ultracentrifuge equipped with schlieren and interference optics (Beckman-RIIC, Glenrothes, Fife, Scotland, U.K.). The dioxygenase (0.7 ml; 10 mg) was dialysed for 18 h at 4°C against 0.1 M-Tris/HCl buffer, pH 7.3, containing 0.05% (w/v) sodium dodecyl sulphate and 0.05% (v/v) mercaptoethanol. At the end of this time dissociation had occurred and the solution was only faintly pink. Sedimentation-velocity data on the dissociated sample were obtained at 20°C and 56000 rev./min by using a double-sector cell fitted with sapphire windows. M_r values were determined by using the meniscus-depletion technique (Yphantis, 1964) in a double-sector interference cell fitted with sapphire windows. All ultracentrifuge runs were performed in the dissociating buffer.

Source of materials

All chemicals were obtained from Sigma Chemical Co., Poole, Dorset, U.K., or from BDH Chemicals, Poole, Dorset, U.K., except as stated in the text. Dioxygenase protein enriched with ^{57}Fe was prepared by the method given in Geary & Dickson (1981).

Results and discussion

E.s.r. measurements

Ferredoxin. The protein was reduced by the addition of the flavoprotein followed by NADH in the e.s.r. tube under N_2 . The reduced protein gave the spectrum shown in Fig. 1(a). Some fine structure was noted that indicated the presence of more than one spectrum. This may have been the result of different conformations of the protein caused by local pH changes, which occur on freezing in phosphate buffer (Williams-Smith *et al.*, 1977; Orii & Morita, 1977). The addition of the zwitterionic buffer Mops, which does not change pH on freezing, produced a rhombic spectrum (Fig. 1b) with g-values at 2.026, 1.890 and 1.834. Integration of the signal yielded a value of 0.7 spins/molecule of protein, reflecting a loss of [2Fe-2S] clusters during purification and storage of the protein (Crutcher & Geary, 1979). The spectrum was observed at temperatures up to 155 K and it was saturated with microwave power at 23 K

Fig. 1. E.s.r. spectra of the ferredoxin (5.2 mg/ml), reduced with 7 mM-NADH in the presence of the flavoprotein (1.5 mg/ml) under N_2 : (a) in 25 mM-phosphate buffer, pH 7.0; (b) in 100 mM-Mops buffer, pH 7.0

Conditions of measurement: temperature 30 K; microwave power 20 mW, frequency 9.18 GHz; modulation amplitude 5 mT; modulation frequency 100 kHz.

($P_i = 11$ mW). This temperature-dependence is typical of [2Fe-2S] rather than [4Fe-4S] clusters (see Rupp *et al.*, 1978). Broadening of the spectrum was only noticeable above 100 K, indicating a slower electron-spin relaxation time than in the [2Fe-2S] plant ferredoxins (Gayda *et al.*, 1976) and thus a stronger exchange coupling between the iron atoms of the cluster.

The mid-point reduction potentials (redox potentials) of the proteins were determined by anaerobic titration at pH 7.0 (Fig. 2b). The redox potential of the ferredoxin was estimated as -155 ± 10 mV, which is the highest value so far recorded for a [2Fe-2S] ferredoxin.

Terminal dioxygenase protein. The dioxygenase was reduced by the addition of the flavoprotein and ferredoxin followed by NADH in the e.s.r. tube under N_2 . The spectrum of the reduced protein is similar to that reported by Axcell & Geary (1975). It has considerable rhombic distor-

Fig. 2. Redox titration curves of the iron-sulphur clusters in: (a) the dioxygenase protein (14 mg/ml) in the presence of ferredoxin (0.017 mg/ml) and 0.23 mM- Fe^{2+} ; (b) the ferredoxin (0.6 mg/ml)

The proteins were titrated in 100 mM-Mops buffer, pH 7.0, as described in the Experimental section. Spectra were recorded as indicated in the legends to Figs. 1 and 3, and the amplitudes of the reduced spectra were plotted against applied redox potentials. The data are fitted to theoretical curves for single-electron carriers with mid-point reduction potentials of (a) -110 mV and (b) -155 mV.

tion, with g -values at 2.018, 1.917 and 1.754 (Fig. 3). Integration of the spectrum gave an intensity of 1.83 spins/molecule. The protein therefore appears to contain two $[2\text{Fe-2S}]$ clusters per molecule of M_r 215000. This agrees with the results of chemical analysis and cluster-extrusion experiments (Crutcher & Geary, 1979; Geary & Dickson, 1981), which indicated a $2[2\text{Fe-2S}]$ protein. However, the e.s.r. spectra, recorded over a wide range of temperature and redox potential, showed no evidence of the presence of more than one paramagnetic species. Since the lineshapes of e.s.r. spectra of $[2\text{Fe-2S}]$ clusters are very sensitive to subtle differences in co-ordination geometry (Cammack, 1975), it seems most probable that the clusters are all in sites of identical co-ordination geometry. The simplest explanation of this observation is that the protein is a dimer.

The e.s.r. spectrum of the dioxygenase protein was detected up to 120 K and was saturated with

Fig. 3. E.s.r. spectra of the reduced dioxygenase protein [54 mg/ml, reduced with 7 mM-NADH in the presence of flavoprotein (1.4 mg/ml) and ferredoxin (0.41 mg/ml)] —, Native protein; ----, protein from cells grown on 90%-enriched ^{57}Fe . The spectra are adjusted to the same integrated intensity. Conditions of measurement were as indicated in the legend to Fig. 1, except that the temperature was 25 K.

microwave power at 23 K ($P_i = 42$ mW). This temperature-dependence is again typical of $[2\text{Fe-2S}]$ ferredoxins, and indicates a faster electron-spin relaxation rate than in the ferredoxin. The low average g -value, large g -value anisotropy and large difference in linewidths for the three g -values make this an unusual spectrum. It closely resembles those obtained from 4-methoxybenzoate mono-oxygenase (Twilfer *et al.*, 1981) and from the $[2\text{Fe-2S}]$ 'Rieske' proteins of the quinone-cytochrome c region of electron-transport chains of respiration and photosynthesis (Rieske *et al.*, 1964; Trumpower *et al.*, 1980; Evans, 1982). The latter proteins have g -values 2.01, 1.90 and 1.78 and very high redox potentials (130 to 310 mV).

The mid-point reduction potential of the dioxygenase protein was determined as described above. When the protein was titrated alone, the redox equilibrium with the mediators was established only slowly, as seen by the non-coincidence of the titration curves in the oxidizing and reducing directions. However, when a small quantity of the ferredoxin (which interacts rapidly with the mediators) was added, a reversible titration curve was obtained. The mid-point potential was estimated to be -112 ± 15 mV.

The curve that best fitted the redox titration data was calculated from the Nernst equation with $n = 1$. Thus the two clusters in the dioxygenase protein showed independent redox behaviour. There was no significant difference when the protein was titrated in the presence of Fe^{2+} . Moreover, this value was the same as the average value obtained in the absence of ferredoxin. Thus the mid-point potential of the [2Fe-2S] clusters in the dioxygenase is not significantly affected by interaction with each other or with iron or ferredoxin.

A sample of the dioxygenase enriched with ^{57}Fe (10.8 mg, 120 μl) was reduced as above and a derivative spectrum was obtained (Fig. 3, ----- curve). The spectrum shows hyperfine broadening by the nuclear spin ($I = \frac{1}{2}$) of ^{57}Fe . Simulation of the spectrum indicated a splitting of 1.3 mT, assuming a 1:2:1 splitting and an isotopic enrichment of 80%. This value is similar to that reported by Twilfer *et al.* (1981) for 4-methoxybenzoate mono-oxygenase.

The behaviour of the reduced clusters was investigated under turnover conditions. A sample of the dioxygenase was reduced as above with NADH, flavoprotein and ferredoxin in the presence of 0.054 μmol (approx. 1.5 molar excess) of Fe^{2+} . Re-oxidation of the reduced clusters, in the presence of Fe^{2+} , was investigated in the absence and in the presence of substrate (benzene) by liberating O_2 into an anaerobic reaction mixture followed by rapid freezing. The reaction was started by addition of 3 μl of 4–5-volume H_2O_2 ; O_2 was liberated by traces of catalase, which contaminate the dioxygenase. The sample tube was frozen within 5 s in an isopentane bath at -150°C . A decrease in size of the cluster signal of 18% due to oxidation was apparent. In another experiment, 20 μl of a solution (11.25 mM) of the substrate benzene was also added; the resultant decrease in

the signal of the reduced [2Fe-2S] cluster was 24%. The solution was thawed and incubated at room temperature for several minutes, after which it was re-frozen. The signal from the cluster at this point had increased again to the original value (allowing for dilution). This indicates that the clusters were re-reduced by excess NADH when the O_2 was exhausted owing to the metabolism of benzene.

In the case of oxidized, fully active, 4-methoxybenzoate mono-oxygenase, signals from high-spin Fe(III) were induced by addition of substrates (Twilfer *et al.*, 1981). In the benzene dioxygenase, no such prominent signals were observed. As prepared, the dioxygenase gave a minor signal at $g = 4.3$ indicative of the presence of high-spin Fe^{3+} in a rhombically distorted site. After the addition of Fe^{2+} (approx. 1.5 molar excess) the signal increased slightly; however, no significant changes in this signal were observed on addition of benzene and/or O_2 , or under the turnover conditions described above.

Flavoprotein. Attempts to determine the redox potential of the flavoprotein by e.s.r. titration were unsuccessful. There was no evidence of the appearance of a free-radical signal indicative of the formation of the stable flavin semiquinone during the titration. A similar observation was made for ferredoxin_{TOL} reductase by Subramanian *et al.* (1981).

Re-activation of benzene dioxygenase by Fe^{2+}

Results from re-activation experiments (Table 1) showed that, if the enzyme was preincubated with Fe^{2+} before the assay, it gave a rate marginally higher than that of the control in which the enzyme was re-activated with Fe^{2+} in the reaction mixture (note that, when the enzyme was incubated with Fe^{2+} , the final concentration of Fe^{2+} was only 40 μM in the reaction mixture, compared with 600 μM in the standard assay, indicating a tight binding of Fe to the enzyme). The activity in-

Table 1. Re-activation of the dioxygenase, before its addition to the reaction mixture, by Fe^{2+} in the presence and in the absence of thiol compounds

Reaction mixtures contained, in a total volume of 3 ml of 0.1 M-Tris/HCl buffer, pH 7.5: 30 μl of re-activated protein or 45 μl of unactivated control (50.5 mg/ml), 0.6 mg of ferredoxin, 1.2 mg of flavoprotein, 1.8 μmol of Fe^{2+} (as FeSO_4) added to the control only, and 1 μmol of NADH (added last). Rates are corrected for dilution of re-activated samples. The reaction was initiated by the addition of benzene. Abbreviations: DTT, dithiothreitol, ME, mercaptoethanol.

Incubation mixture	Reaction rate	
	(μmol of O_2/min^*) (% of control rate)	
Dioxygenase + Fe^{2+}	1.08	103
Dioxygenase + Fe^{2+} + DTT	1.39	132
Dioxygenase + Fe^{2+} + ME	1.47	140
Dioxygenase + Fe^{2+} + DTT + ME	1.63	155
Control	1.05	100

* 1 ml of air-saturated buffer at $30^\circ\text{C} \equiv 245 \text{ nmol}$ of O_2 (Glasstone, 1946).

creased much more if either dithiothreitol or mercaptoethanol was present in the re-activation mixture, and was maximal when both thiol compounds were present. The number of iron atoms bound per molecule of protein was measured in samples re-activated with this mixture as described in the Experimental section (Table 2). After column chromatography it was found that 2.1 Fe atoms (average of two runs) per molecule of protein were bound in addition to cluster iron. The increase in activity after this treatment when compared with that of a control activated in the reaction mixture was of the same order (160%; average of two runs) as was obtained in re-activation experiments, indicating that iron was not lost to an appreciable extent from the binding site during chromatography and subsequent concentration (Table 2). Thus, if this represents the fully re-activated protein, it contains two iron atoms per dimeric molecule.

Proteins thus re-activated showed no change in the characteristics of the $g = 4.3$ e.s.r. signal described above. We infer from these results that, in its active form, the mononuclear Fe site of benzene dioxygenase is e.s.r.-silent and that, during catalysis, e.s.r.-active species are either not generated or are present at low equilibrium concentrations that are not detected. Further work is necessary, however, to confirm this.

Ultracentrifuge measurements

Investigations of the subunit composition of the dioxygenase by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate gave unsatisfactory results, only broad bands being obtained. Therefore the structure was investigated by ultracentrifuge measurements in the presence of sodium dodecyl sulphate. Examination of the dioxygenase, after dissociation, by the sedimentation-velocity method with schlieren optics indicated that dissociation was almost complete. A

small peak corresponding to undissociated dioxygenase sedimented first, followed by a single symmetrical peak, with a small leading shoulder thought to represent a minor contaminant. This peak had cleared the meniscus by the end of the run (2.5 h), and no smaller material was present. The subunit peak showed no tendency to resolve into more than one component, and $s_{\text{obs.}}$ was calculated to be $3.45 \times 10^{-13} \text{ s}^{-1}$.

The M_r of the dissociated material was determined by the meniscus-depletion (Yphantis equilibrium) technique to be 47000 ± 1000 (average of three determinations). No evidence was found for inhomogeneity or the presence of lighter material in the sample.

The dioxygenase protein has M_r 215000. One possible interpretation of the results is that it consists of four equivalent subunits, although the subunit M_r is somewhat lower than expected. The alternative is two pairs of closely related subunits ($\alpha_2\beta_2$ structure) of similar M_r . Within the resolution of the ultracentrifuge experiments, it would not be possible to distinguish two types of subunits differing in molecular mass by less than 5000 Da. The $\alpha_2\beta_2$ structure is consistent with the analyses, which indicate two [2Fe-2S] clusters and two iron atoms bound per molecule, and therefore seems more probable.

E.s.r. studies on the dioxygenase component of benzene dioxygenase have demonstrated similarities between this enzyme and 4-methoxybenzoate mono-oxygenase (Twilfer *et al.*, 1981), benzoate 1,2-dioxygenase (Yamaguchi & Fujisawa, 1982) and pyrazon dioxygenase (Sauber *et al.*, 1977), despite differences in substrate specificity, M_r and intermediate electron-carrier protein. The values for the redox potentials of both iron-sulphur proteins of benzene dioxygenase are exceptionally high for [2Fe-2S]-cluster-containing proteins.

Spin integration has confirmed the presence of

Table 2. Iron content and relative activity of the dioxygenase after re-activation under reducing conditions and chromatography. Conditions for re-activation are described in the Experimental section. Reaction mixtures for assay were similar to those described in Table 1, except that 45 μl samples of protein solution were used throughout. Fe^{2+} was added to reaction mixtures to obtain the pre-column rate.

Analysis	Run 1	Run 2
Protein concentration pre-column	50.5 mg/ml	64.5 mg/ml
Protein concentration post-column	35.5 mg/ml	22.3 mg/ml
Total Fe content pre-column	3.98/mol	3.76/mol
Total Fe content post-column	6.15/mol	5.79/mol
Iron bound (non-cluster)	2.17	2.03
Average Fe^{2+} /mol		2.1
Pre-column rate (μmol of O_2 /min per mg)	0.4	0.42
Post-column rate (μmol of O_2 /min per mg)	0.6	0.72
Percentage of control rate	150%	171%
Average percentage of control rate		160.5%

two [2Fe-2S] clusters in the dioxygenase. The presence of two additional Fe^{2+} atoms in the fully active protein molecule in conjunction with the apparently symmetrical subunit structure suggests that the active site contains $[\text{2Fe-2S}]\text{Fe}^{2+}$ as the oxygen-activating unit, similar to that found by Bill *et al.* (1981) for 4-methoxybenzoate mono-oxygenase, and that each molecule contains two active sites. This view is borne out by the fact that no distinction can be made between clusters on the basis of their e.s.r. spectra or their redox potentials.

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