Biochem. J. (1981) **195**, 199–203
Printed in Great Britain

Mössbauer spectroscopic studies of the terminal dioxygenase protein of benzene dioxygenase from *Pseudomonas putida*

Philip J. GEARY* and Dominic P. E. DICKSON†
**Shell Research Ltd., Shell Biosciences Laboratory, Sittingbourne Research Centre, Sittingbourne, Kent ME9 8AG, U.K., and †Department of Physics, University of Liverpool, Liverpool L69 3BX, U.K.*

(*Received 7 October 1980/Accepted 5 January 1981*)

Mössbauer spectra obtained from the terminal dioxygenase protein of the benzene dioxygenase system from *Pseudomonas putida* show that it contains [2Fe–2S] centres similar to those of the two-iron plant-type ferredoxins. In the oxidized form the two iron atoms within the centre are high-spin ferric but with considerable inequivalence. In the reduced form the centre contains one extra electron, and this is localized on one of the iron atoms, which becomes high-spin ferrous.

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*-1,2-dihydroxycyclohexa-3,5-diene (*cis*-benzene glycol) in the presence of NADH and Fe^{2+} (Axcell & Geary, 1975). The system consists of a flavoprotein, an intermediate electron-transfer protein, similar to a two-iron ferredoxin, and a terminal dioxygenase, which is also an iron–sulphur protein and contains four atoms each of iron and inorganic sulphur (Crutcher & Geary, 1979).

Results from titration and cluster-extrusion experiments with the terminal dioxygenase indicate that the protein contains two [2Fe–2S] electron-transport centres (Crutcher & Geary, 1979). However, the extrusion data are not entirely unambiguous, and the titration results could be indicative of one [4Fe–4S] centre capable of undergoing a two-electron reduction (B. A. Averill, personal communication). The u.v. and visible spectra are characteristic of the [2Fe–2S] centres of the plant-type ferredoxins.

Mössbauer spectroscopy, which depends upon the chemical state and environment of the atoms containing the ^{57}Fe Mössbauer nuclei, yields characteristic spectra for the two types of iron–sulphur centre, particularly when they are examined in both their oxidized and reduced states (Cammack *et al.*, 1977). ^{57}Fe was incorporated into the iron–sulphur centres of the terminal dioxygenase protein by growing the cells on a medium containing iron in this isotopic form.

Experimental

Growth of Pseudomonas putida enriched with ^{57}Fe

Cells of *Pseudomonas putida* were grown with benzene as the sole source of carbon by the method of Axcell & Geary (1973). Growth was started with ordinary $FeSO_4$ as the iron source (natural iron contains only 2% of ^{57}Fe) and allowed to proceed for 24h, with harvesting twice. Batch culture was used, the fermenter being drained at each harvest and refilled with fresh sterile medium. Residual cells formed the inoculum for the subsequent batch. For growth of the third batch iron in the medium was supplied as ^{57}Fe, added to the medium as a solution containing 20mg of ^{57}Fe foil (Atomic Energy Research Establishment, Harwell, Abingdon, Oxon, U.K.) dissolved in 0.6ml of 1.5M-H_2SO_4 and diluted to 3ml with sterile distilled water. A total of 400g (wet weight) of cells was obtained over a period of 48h, with harvesting four times. A solution containing a further 20mg of ^{57}Fe was added to each batch before growth.

Preparation of benzene dioxygenase for Mössbauer studies

The three component proteins of benzene dioxygenase were purified by the method of Crutcher & Geary (1979). The fraction containing the flavoprotein was further purified by adding an excess of FAD in the form of the disodium salt (Sigma Chemical Co., Kingston upon Thames, Surrey, U.K.) (5mg per 100mg of protein), followed by chromatography on a column (80cm × 3cm²) of Sephadex G-150 (superfine grade) (Pharmacia, Uppsala, Sweden) equilibrated and eluted with 25mM-potassium phosphate buffer, pH 7.3, to remove unbound FAD and the pyrocatechase contaminant. The flavoprotein was located by the 2,6-dichlorophenol-indophenol assay (Axcell & Geary, 1975) and concentrated by using an

0306-3275/81/040199-05$01.50/1

Amicon ultrafiltration cell with a PM-10 membrane (Amicon, High Wycombe, Bucks., U.K.). Only the terminal dioxygenase was prepared from cells grown with ^{57}Fe as the source of iron. In order to obtain an optimized absorption intensity in the Mössbauer spectrum the 2 $cm^2 \times 0.5$ cm sample should contain approx. 100 μg of ^{57}Fe, which is equivalent to 100 mg of the dioxygenase protein, which has 4 iron atoms per molecule (relative molecular mass 215300; Crutcher & Geary, 1979). To achieve these quantities, 400 g of cells were processed in two batches, which were combined at the final Sephadex G-150 stage, which yielded 180 mg of >90%-pure protein in a volume of 2 ml. Assays showed an equivalence of the iron and inorganic sulphur content of the terminal dioxygenase at this stage, which demonstrates that all of the iron was incorporated into the iron–sulphur centres. The Mössbauer experiments were performed with the protein as thus prepared and containing no additional iron.

To demonstrate oxygenase activity towards benzene it is necessary to add Fe(II) to reaction mixtures containing the purified enzyme components, since the Fe(II) essential for this function is removed from the dioxygenase during purification. The terminal dioxygenase protein has been shown to bind up to 0.8 additional atom of iron per molecule of protein after incubation at pH 6.6 (100 mM-succinate/NaOH buffer) with a 2–5-fold molar excess of $FeSO_4$ and removal of unbound iron by gel filtration on a column (22 cm $\times$ 0.8 cm^2) of Sephadex G-25 (superfine grade) (Pharmacia). Such preparations showed up to 60% of maximum activity when assayed in the absence of additional $FeSO_4$ (Crutcher & Geary, 1979; P. J. Geary, unpublished work).

The oxidized form of the terminal dioxygenase, as prepared above, was transferred without further treatment to a nylon Mössbauer cell (internal dimensions 2 $cm^2 \times 0.5$ cm), frozen at −22°C and stored in liquid N_2. To a further 1 ml of the solution, which had been gassed with N_2, was added 6 mg of solid NADH (Sigma Chemical Co.) followed by 80 μl (0.3 mg) of the intermediate electron-transfer protein. (This protein contained natural iron in its centres, so that it would not interfere with the spectra from the terminal dioxygenase protein.) Reduction of the iron–sulphur centres of the terminal dioxygenase, which proceeds independently of additional Fe(II) and hence of oxygenase activity (Crutcher & Geary, 1979), was initiated by the addition of 30 μl (0.3 mg) of the flavoprotein treated as described above (NADH–intermediate electron-transfer protein oxidoreductase). The mixture was incubated at 20°C under N_2 until no further bleaching of the red colour attributable to the iron–sulphur centres of the dioxygenase was noted. A further 2 mg of solid NADH was then added to ensure complete reduction of the sample, and the mixture was rapidly transferred to a Mössbauer cell and frozen as before.

Mössbauer spectroscopy

The Mössbauer spectra were obtained with a conventional constant-acceleration spectrometer with a 50 mCi source of ^{57}Co in a rhodium matrix. The spectra are plotted with the centre of the spectrum of iron metal at room temperature (293 K) as the zero of the velocity axis. Where appropriate, the spectra were computer-fitted to quadrupole-split doublet components, with the chemical shift, quadrupole splitting, linewidth and intensity of each component being variables in the fitting procedure.

Results and discussion

The spectrum of the oxidized protein at 195 K (Fig. 1*a*) consists of two equal-intensity quadrupole-split doublet components, both of which have Mössbauer parameters (see Table 1) that are characteristic of high-spin ferric atoms with tetrahedral sulphur co-ordination. These parameters are similar to those obtained from various two-iron ferredoxins in the oxidized state (see, e.g., Cammack *et al.*, 1971; Rao *et al.*, 1971; Dunham *et al.*, 1971). A noteworthy feature of this spectrum is the degree of resolution of the two components, which are of equal intensity and have narrow linewidths; this indicates that there are two distinct Fe^{3+} sites in equal numbers. Although there is clearly some structure observable in the quadrupole-split doublet spectra of the oxidized form of the various two-iron ferredoxins that have been investigated so far, only with the oxidized ferredoxin from *Halobacterium*, the halophilic bacterium from the Dead Sea, is there a degree of resolution of the two components that is comparable with that observed in the present case

Table 1. *Mössbauer parameters of the terminal dioxygenase protein of benzene dioxygenase from Pseudomonas putida*

The chemical shifts δ (relative to pure iron metal at room temperature) and the quadrupole splittings Δ were determined by least-squares computer fitting and are given in mm/s. Errors are ± 0.01 mm/s for the chemical shifts and ± 0.02 mm/s for the quadrupole splittings.

	Temperature (K)	Component	δ	Δ
Oxidized	195	Fe^{3+} 1	0.18	0.44
		Fe^{3+} 2	0.29	1.03
	77	Fe^{3+} 1	0.23	0.45
		Fe^{3+} 2	0.33	1.03
Reduced	195	Fe^{3+}	0.25	0.70
		Fe^{2+}	0.68	2.94

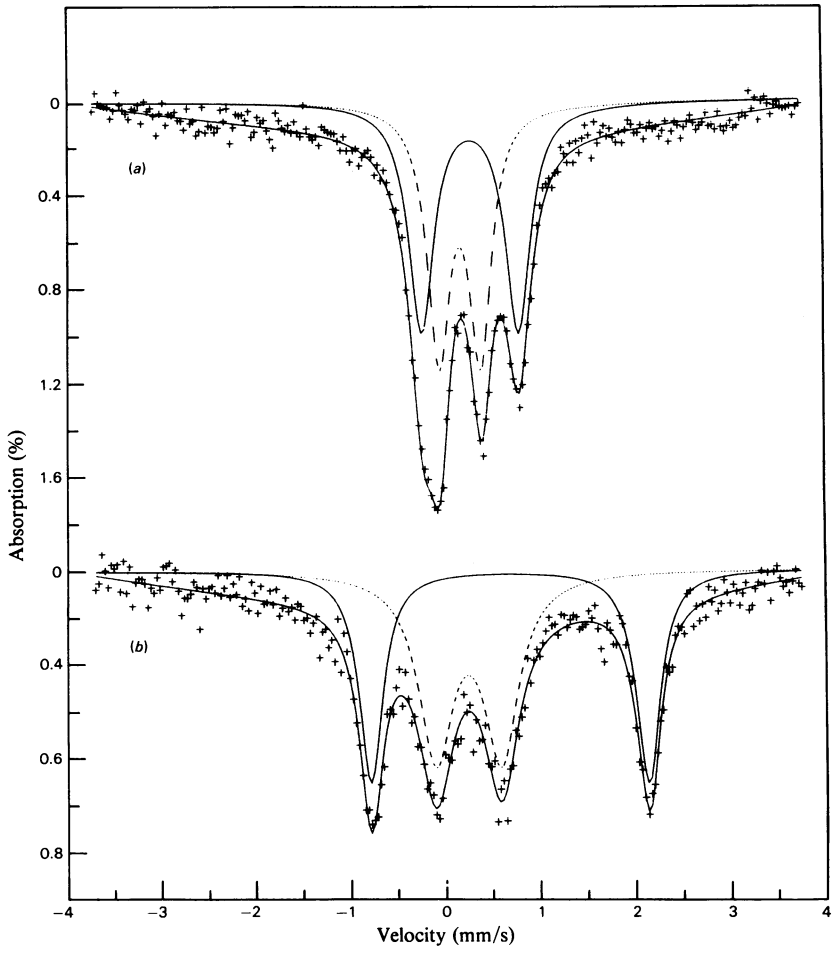

Fig. 1. *Mössbauer spectra of the terminal dioxygenase protein at 195 K*

For experimental details see the text. (*a*) Oxidized; component 1 is shown by the broken line and component 2 by the continuous line. (*b*) Reduced; the Fe^{3+} component is shown by the broken line and the Fe^{2+} component by the continuous line.

(Werber *et al.*, 1978). There are two possible reasons for this marked inequivalence of the two Fe^{3+} sites. Either the two iron–sulphur centres within each molecule are inequivalent, or alternatively, and much more likely, the two iron atoms within each centre are inequivalent. The latter explanation is consistent with the observation of only a single Fe^{3+} component in the spectrum of the reduced protein (see below).

The spectrum of the reduced protein at 195 K (Fig. 1*b*) also consists of two quadrupole-split doublets of equal intensity. One component has parameters (see Table 1) similar to those of the two components of the spectrum of the oxidized protein and therefore characteristic of high-spin ferric atoms. The other component has parameters characteristic of high-spin ferrous atoms with tetrahedral sulphur co-ordination, as observed in the spectra of reduced two-iron ferredoxins (see, e.g., Cammack *et al.*, 1971; Rao *et al.*, 1971). Thus it appears that, of the two electrons that go on to each molecule of the protein on reduction, one goes to each two-iron centre and within that centre reduces one of the Fe^{3+} atoms to Fe^{2+}. The other Fe^{3+} atom is also marginally affected by the change in the electronic structure of the centre on reduction, as witnessed by the fact that Mössbauer parameters of the Fe^{3+} component in the spectrum of the reduced protein are different from the Mössbauer parameters of either component in the spectrum of the oxidized protein.

The behaviour described above, with essentially two Fe^{3+} atoms in the oxidized state and one Fe^{3+} atom and one Fe^{2+} arom in the reduced state, as

illustrated clearly in Fig. 1, is characteristic of two-iron ferredoxins. This behaviour is markedly different from what is observed in the ferredoxins with four-iron centres, in which there is considerable electron delocalization between the iron atoms, the extra reducing electron is shared and there is no evidence for any high-spin ferrous component in the spectra (Cammack *et al.*, 1977). The spectra of Fig. 1 therefore constitute strong evidence that the terminal dioxygenase protein contains [2Fe–2S] centres.

The spectra of the oxidized and the reduced protein at 77 K are shown in Fig. 2. The spectrum of the oxidized protein is closely similar to that obtained at 195 K except for the small change in the chemical shift that results from the second-order Doppler effect. The 4.2 K spectrum (not shown) is also similar. Thus the low-temperature spectra of the oxidized protein consist of quadrupole-split doublet components with no evidence for any magnetic hyperfine interaction, as would be expected from isolated Fe^{3+} atoms at low temperatures when the spin relaxation is slow. This diamagnetic behaviour results from antiferromagnetic coupling between the spins of the two Fe^{3+} atoms, which gives the centre a net spin of zero. This behaviour is observed in ferredoxins with both two-iron and four-iron centres. On reduction, the centre gains an extra electron and the antiferromagnetic coupling yields a centre with a spin of $\frac{1}{2}$. At high temperatures the direction of this spin is changing rapidly with time as a result of spin–lattice relaxation, the magnetic hyperfine interaction averages to zero and only the quadrupole interaction is observed in the Mössbauer spectrum, as seen in the spectrum of the reduced protein at 195 K (Fig. 1*b*). At lower temperatures the spin–lattice relaxation slows down and the magnetic hyperfine interaction leads to a further splitting of the Mössbauer spectrum. This can be seen in the 77 K spectrum of the reduced protein (Fig. 2*b*). This spectrum is very similar to that of the reduced two-iron ferredoxins at low temperatures (see, e.g., Rao *et al.*, 1971). It should be noted that the temperature at which the magnetic splitting becomes observable is different in the different two-iron ferredoxins, as a result of the effects on the

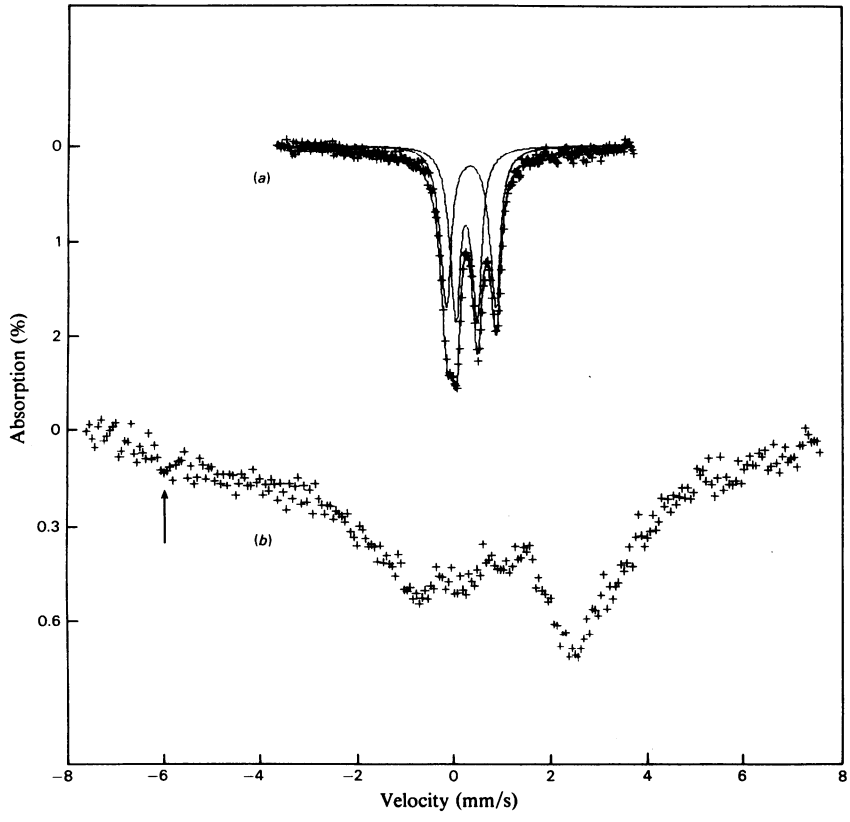

Fig. 2. *Mössbauer spectra of the terminal dioxygenase protein at 77 K*
For experimental details see the text. (*a*) Oxidized. (*b*) Reduced.

spin–lattice relaxation processes of small changes in the conformation of the centre. The close correspondence between the spectrum shown in Fig. 2(*b*) and the spectra of reduced two-iron ferredoxins at low temperatures is additional strong evidence that the present protein contains two-iron centres. Reduced four-iron ferredoxins also show magnetic hyperfine interactions in their low-temperature Mössbauer spectra (Mullinger *et al.*, 1975), but with a significantly different spectral profile. In addition, because of the increased electron delocalization, as discussed above, the magnetic hyperfine coupling constants are smaller and the overall splitting is decreased. Thus in the low-temperature spectra of reduced four-iron ferredoxin the characteristic lowest-energy line is at −4 mm/s, whereas in reduced two-iron ferredoxin the equivalent line is at −6 mm/s, which is where this line (shown arrowed in Fig. 2*b*) is to be found in the 77 K spectrum of the reduced terminal dioxygenase protein. Thus the low-temperature spectrum of the reduced protein provides considerable confirmatory evidence that it contains [2Fe–2S] centres rather than [4Fe–4S] centres.

Similar results have been obtained in recent work on the iron–sulphur protein putidamono-oxin, which is part of the multi-enzyme system 4-methoxybenzoate *O*-demethylase from *Pseudomonas putida* (Bill *et al.*, 1980).

The nature of the additional iron required for activity is an intriguing question. Whether it is in any way related to the iron–sulphur centres is open to speculation and awaits further work. However, the substantial inequivalence of the two iron atoms within the oxidized centre could be related to distortion of this centre associated with the ability of the additional Fe(II) to come sufficiently close to effect oxygenase activity through a transfer of electrons to bound oxygen.

D. P. E. D. gratefully acknowledges receipt of a Nuffield Foundation One-Year Science Research Fellowship.

References

Axcell, B. C. & Geary, P. J. (1973) *Biochem. J.* **136**, 927–934

Axcell, B. C. & Geary, P. J. (1975) *Biochem. J.* **146**, 173–183

Bill, E., Bernhardt, F. H., Marathe, V. R. & Trautwein, A. (1980) *J. Phys.* (*Paris*) **41**, C1-485–C1-486

Cammack, R., Rao, K. K., Hall, D. O. & Johnson, C. E. (1971) *Biochem. J.* **125**, 849–856

Cammack, R., Dickson, D. P. E. & Johnson, C. E. (1977) in *Iron–Sulfur Proteins* (Lovenberg, W., ed.), pp. 283–330, Academic Press, New York

Crutcher, S. E. & Geary, P. J. (1979) *Biochem. J.* **177**, 393–400

Dunham, W. R., Bearden, A. J., Salmeen, I. T., Palmer, G., Sands, R. H., Orme-Johnson, W. H. & Beinert, H. (1971) *Biochim. Biophys. Acta* **253**, 134–141

Mullinger, R. N., Cammack, R., Rao, K. K., Hall, D. O., Dickson, D. P. E., Johnson, C. E., Rush, J. D. & Simopoulos, A. (1975) *Biochem. J.* **151**, 75–83

Rao, K. K., Cammack, R., Hall, D. O. & Johnson, C. E. (1971) *Biochem. J.* **122**, 257–265

Werber, M. M., Bauminger, E. R., Cohen, S. G. & Ofer, S. (1978) *Biophys. Struct. Mech.* **4**, 169–177.