***Pseudomonas* cytochrome c-551 peroxidase**

A purification procedure and study of CO-binding kinetics

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A procedure is described for the purification of cytochrome *c* peroxidase from *Pseudomonas aeruginosa* involving extraction by sonication, followed by acid precipitation and chromatography on only two types of gel. The final preparation had a purity ratio A_{407}/A_{280} of 4.2, and was found to be essentially pure by isoelectric focusing. The enzyme was shown to be unstable during degassing under vacuum except in the presence of detergent. The kinetics of CO binding to dithionite-reduced peroxidase were studied with stopped-flow and flash-photolysis techniques, and the results obtained between pH 5 and 7 suggest the existence of two forms of dithionite-reduced enzyme in slow equilibrium.

Pseudomonas cytochrome *c*-551 peroxidase (EC 1.11.1.5) is an enzyme which catalyses the reduction of H_2O_2 using electrons donated by either *Pseudomonas* cytochrome *c*-551 or the *Pseudomonas* blue copper protein, azurin. Unlike other peroxidases the protein has been found to possess two haem *c* components in a single polypeptide chain (Ellfolk & Soininen, 1971), the total molecular mass being approx. 44 kDa (Soininen & Ellfolk, 1973*b*). A number of studies by Ellfolk and co-workers have shown that the two haem centres differ in both structure and function (Rönnerberg *et al.*, 1979; Rönnerberg & Ellfolk, 1979). At room temperature, resonance Raman spectra show that the two haems have different spin states (Rönnerberg *et al.*, 1980). The likely mechanism of peroxidation requires the high-spin haem centre to be the site of peroxide attack, whereas the low-spin haem is thought to be the component which reacts directly with the physiological electron donors.

It has been known for some time that the dithionite-reduced form of the enzyme forms a complex with CO (Soininen & Ellfolk, 1973*a*), and more recently it was discovered that only the high-spin haem component provides a ligand-binding site (Rönnerberg & Ellfolk, 1979). In this paper we describe some rapid-reaction kinetic studies of the CO-binding process. We also give details of a purification procedure adapted from those previously published, resulting in a more convenient method of isolation of the enzyme.

Materials and methods

All chemicals were of Analar grade and were obtained from BDH, with the following exceptions: $FeCl_3 \cdot 6H_2O$ and $MgSO_4 \cdot 7H_2O$ from Fisons, Loughborough, Leics., U.K.; Tris and Tween 80 from Sigma; powdered yeast extract from LabM, Mark Lane, London, U.K. O_2 -free N_2 and CO gases were obtained from B.O.C., London SW19, U.K. and were dispensed from the cylinders and stored in glass vessels over an alkaline solution of dithionite-reduced anthraquinone sulphate before use. A mixture of O_2/N_2 (1:19) for the aeration of cultures was supplied by B.O.C. Special Gases, London SW19, U.K.

Concentrations of the enzyme were calculated using $\epsilon_{407}^{ox} = 237 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (Soininen & Ellfolk, 1973*a*). Ferrocycytochrome *c*-551 was prepared by the addition of solid sodium dithionite to cytochrome *c*-551, excess reductant being removed on a column of Sephadex G-25, and was assayed spectrophotometrically using $\Delta\epsilon_{350}(\text{red.} - \text{ox.}) = 20.0 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (Ambler, 1963). Stock H_2O_2 solutions were assayed by titration against acidified $KMnO_4$. Dithionite solutions were prepared anaerobically in a sealed Thunberg tube by dissolving solid sodium dithionite, placed in a sidearm, only after the buffer had been equilibrated thoroughly with O_2 -free N_2 .

Assays of peroxidative activity were performed at room temperature, 20°C. A 5–25 μl sample of enzyme solution was added to 2.5 ml of 0.01 M-

potassium phosphate buffer, pH 6.0, containing approx. 12 μ M-ferrocytochrome *c*-551, in a 1 cm path-length cuvette. The reaction was initiated by the addition of H₂O₂ to a concentration of 78 μ M, and the oxidation of ferrocytochrome *c*-551 was monitored at 550 nm. Measurements were made at 550 nm rather than 551 nm because of the shift in position of the α band of cytochrome *c*-551 at acid pH (Silvestrini *et al.*, 1981). From the observed initial rate was subtracted the basal rate of oxidation measured before the addition of peroxide. Enzymic activity was expressed in units of μ mol of ferrocytochrome *c*-551 oxidized/min per ml of enzyme solution.

Absorption spectrophotometry was carried out on either a Cary 118C or a Pye– Unicam SP8-200 instrument. Isoelectric focusing was performed on an LKB Ampholine PAG-plate over the pH range 3.5–9.5 in accordance with the manufacturer's recommendations. The gel was fixed for 1 h in a solution containing 11.5% trichloroacetic acid and 3.45% sulphosalicylic acid, and then washed for 5 min in a destaining solution consisting of ethanol/acetic acid/water (25:8:67, by vol.). The plate was stained for 10 min at 60°C with 0.115% Coomassie Brilliant Blue R-250 dissolved in destaining solution, followed by destaining with several changes of solution until the background was clear. The gel was then photographed without further preserving steps.

Rapid-reaction experiments

Stopped-flow measurements were made using an apparatus similar to that described by Gibson & Milnes (1964), containing a 2 cm path-length cell. The analogue output from a log amplifier was sampled at programmed intervals by an A/D converter coupled to a PDP 8/a minicomputer. Reaction trace sampling, and displays of the sampled data on a Tektronix type 564 storage oscilloscope, were controlled by means of user-defined functions in Lab 4V BASIC subroutines obtained from S. R. Vivian, University of Manitoba, Winnipeg, Canada. These subroutines were also used to project semi-logarithmic plots during the analysis of trace data that had been stored on floppy discs. Programs written in this laboratory allow for trace data to be analysed as a single exponential decay, or as a sum of up to three exponential decays. For the more complex reaction traces, analysis could be performed either using a modification of the general least squares adjustment program described by Wilkins *et al.* (1974), or by an automated 'peeling' procedure using a linear regression method to determine the reaction rates and amplitudes of the linear portions of successive semi-logarithmic plots (Gutfreund, 1972). Stopped-flow experiments were performed anaerobically, with all solutions to be

mixed being transferred in gas-tight syringes and containing 0.5 mM-dithionite.

An Applied Photophysics flash photolysis apparatus capable of dissipating up to 200 J was used to study CO recombination after photodissociation. Trace data from these experiments were initially stored in a Datalab 905 transient recorder before being passed to the computer for storage on floppy discs. Trace analysis, after conversion to values of absorbance change, was carried out in a manner similar to that described for stopped-flow data. The sealed flash cell, of volume 2.3 ml and pathlength 1 cm, could also be scanned in the Cary 118C spectrophotometer in order to measure static absorption spectra.

All enzyme solutions for use in kinetic experiments were degassed under vacuum and equilibrated with O₂-free N₂ in the presence of 0.01% (v/v) Tween 80. This concentration of detergent is sufficient to prevent the drastic loss of absorbance and of activity caused by degassing in the absence of Tween 80, yet does not affect the rate of the peroxidative reaction when added to the assay mixture.

Cultivation of *Ps. aeruginosa*

Samples of *Ps. aeruginosa* (N.C.T.C. 6750) were supplied by the National Collection of Type Cultures, Colindale, London, U.K. In a typical cultivation 40 l of growth medium (Ellfolk & Soininen, 1970), in a stainless steel barrel fitted with a New Brunswick head, was inoculated with 200 ml of a starter culture and a few drops of silicone anti-foaming agent were added. The contents of the barrel were stirred and aerated with a mixture of O₂/N₂ (1:19) during growth. The pH of the culture, which tends to rise during cultivation, was maintained between 6.9 and 7 by means of a pH-stat device automatically delivering 5% orthophosphoric acid as required. Automated pH control during growth results in improvements in both cell yield and peroxidase content over cultures where the pH was allowed to rise. Growth ceases after approx. 18 h at 37°C. After rapid cooling of the contents of the barrel to room temperature, the cells were collected by continuous-flow centrifugation using an SS-34 rotor at 18000 rev./min in a Sorval RC-5B centrifuge, and pumping at a rate of approx. 15 l/h. Typically, a 40 l culture yielded 140–160 g of wet cell paste, which was stored at –20°C until needed.

Purification of the enzyme

All steps of the procedure were performed at 2°C or in ice. After being allowed to thaw, 250 g of *Ps. aeruginosa* cell paste was stirred into 2 litres of 0.02 M-sodium phosphate, pH 7. Portions (150 ml) of the suspension were sonicated in ice, using a Dawes

Soniprobe at 100 W power, for 90 s. The release of peroxidase activity was found not to increase with further sonication. The sonicate was centrifuged at 23 000 *g* for 50 min, and the pellet of cell debris discarded to leave the crude extract.

The pH of the crude extract was then lowered to 4.7 by the addition of 1 M-acetic acid, and the extract was stirred for 20 min. The precipitate, collected by centrifugation at 9000 *g* for 60 min, was redissolved in 0.02 M-sodium phosphate, pH 7.0, by stirring overnight, with additions of 0.1 M- Na_2HPO_4 to give a final pH of 6.6 and a volume of 410 ml. The redissolved acid precipitate was then clarified by centrifugation at 9000 *g* for 30 min.

The next purification step was by batch chromatography on CM-Sephadex C-50. The preparation was adjusted, by the addition of NaH_2PO_4 and distilled water, to the same pH and conductivity as 0.01 M-sodium phosphate, pH 6.0. To this was added 250 ml of a slurry of CM-Sephadex C-50 equilibrated in the same buffer. This mixture was stirred for 30 min, and then allowed to settle for 20 min. The supernatant, which still contained a significant amount of peroxidase activity, was siphoned off from the gel and the procedure was repeated using a further 150 ml of slurry. The two batches of gel, to which was bound most of the enzyme, were then combined and used to make a CM-Sephadex column measuring 4 cm $\times$ 20 cm. The column was washed with 2 column vol. of the equilibrating buffer and then eluted with 0.02 M-sodium phosphate, pH 6.8. The coloured fractions were pooled, since at this stage cytochrome *c*-551 peroxidase is the only coloured protein present in any significant amount.

The pool was concentrated from 108 ml to 25 ml by ultrafiltration, using an Amicon apparatus with a

PM 10 membrane under a nitrogen pressure of 172.5 kPa (25 lbf/in²). This concentrated pool was run overnight under hydrostatic pressure on a column (5.1 cm $\times$ 68 cm) of Sephadex G-100 equilibrated in 0.025 M-sodium phosphate, pH 6.0. Fractions (approx. 6 ml) were collected, and those with an absorbance ratio A_{470}/A_{280} greater than 3.0 were combined. This band was found to correspond to the peak of enzyme activity.

The Sephadex G-100 pool was diluted to the conductivity of 0.01 M-sodium phosphate, at pH 6.0, and added to a column (1.5 cm $\times$ 14.5 cm) of CM-Sephadex C-50 equilibrated in the same buffer. The column was washed with 100 ml of the equilibrating buffer, followed by 100 ml of 0.01 M-sodium phosphate, pH 6.4, which caused the tightly bound red-brown band of enzyme to become diffuse. The enzyme was eluted with 0.02 M-sodium phosphate, pH 6.8, in a volume of 24 ml.

Spectrophotometric measurements, cytochrome *c*-551 peroxidase activities, and protein concentrations at various stages in the preparation are shown in Table 1. The technique of analytical isoelectric focusing was used to demonstrate that the purified enzyme was virtually free of contaminating proteins (Fig. 1).

The yield of enzyme, relative to the crude extract, was 32% and the enzyme had a purity ratio A_{407}/A_{280} of 4.2.

Results

Reaction between dithionite-reduced enzyme and CO

The reaction was examined using both flash photolysis and stopped-flow techniques under pseudo-first-order conditions. The regeneration of

Table 1. Details of a representative purification of cytochrome *c* peroxidase from 250 g of *Ps. aeruginosa* cell paste

Purification stage	Volume (ml)	Activity		Protein concn. (mg/ml)	Specific activity (units/mg)	Enrichment (-fold)	Recovery (%)	A_{407}	A_{407}/A_{280}
		(Units/ml)	(Total units)						
1. Crude cell-free extract	1970	3.34	6580	7.4*	0.45	(1)	(100)	—	—
2. Redissolved ppt. from acid precipitation	410	13.0	5330	20.6*	0.63	1.4	81	—	—
3. Pooled fractions from first CMC-50 column	108	29.1	3140	2.8*	10.4	23.1	48	1.49	1.0
4. Pooled fractions from Sephadex G-100 column	88	24.6	2165	0.34†	72	160	33	1.26	3.2
5. Pooled fractions from second CMC-50 column	24	87.9	2110	0.65†	135	300	32	3.42	4.2

* Measured by the biuret method (Gornall *et al.*, 1949).

† Measured spectrophotometrically (Warburg & Christian, 1941).

Fig. 1. Polyacrylamide-gel isoelectric focusing of samples from different stages of the isolation of *Pseudomonas cytochrome c peroxidase*

(a) Pooled fractions from first CMC-50 column; (b) pooled fractions from Sephadex G-100 column; (c) pooled fractions from second CMC-50 column.

the ferrocycytochrome *c*-551 peroxidase-CO complex, following displacement of the combined CO by a short photolysis flash, was observed to be a single exponential process throughout the range of CO concentrations employed (10–1000 μM). However, the reaction between dithionite-reduced enzyme and CO (10–70 μM), examined using the stopped-flow apparatus, comprised two processes occurring at well separated rates.

Effect of CO concentration

The pseudo-first-order rate of recombination after flash photolysis, measured at 415 nm, showed a linear variation with CO concentration at pH 6 (Fig. 2a), giving a second-order rate constant of $1.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. The extent of the absorbance change did not depend upon CO concentration. Similar results were obtained when the experiment was repeated in the absence of Tween, although the necessary degassing caused the measured absorbances to be smaller.

Fig. 2. [CO] dependence of the pseudo-first-order rate of regeneration of CO-ferrocycytochrome *c*-551 peroxidase after flash photolysis (a), and comparison of pseudo-first-order rates of complex regeneration after flash photolysis (O) and of the faster process observed during the reaction of dithionite-reduced enzyme and CO in the stopped-flow apparatus (●) (b)

The buffer was $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, $I = 0.05$, pH 6, containing 0.01% (v/v) Tween 80. The enzyme concentration was 1.23 μM for flash photolysis, and 0.74 μM after mixing for stopped-flow experiments. Temperature 20°C, observation wavelength 415 nm. In repeat experiments, values of k were reproducible to better than $\pm 5\%$.

In the corresponding stopped-flow experiment at pH 6, the rates of the faster process were very close to those determined at comparable CO concentrations after flash photolysis (Fig. 2b). The rate of the slower process was independent of the CO concentration; the first-order rate constant was 0.46 s^{-1} . The extents of both kinetic phases remained constant throughout the range of CO concentrations.

Effect of pH

The rate of recombination after photodecomposition was examined at a range of pH values from 5 to 9, in a series of buffers of equal ionic strength

Table 2. Variation with pH of pseudo-first-order rates of CO-ferrocycytochrome *c*-551 peroxidase regeneration after flash photolysis, and of CO binding by dithionite-reduced enzyme in the stopped-flow apparatus

All buffers were of ionic strength $I = 0.05$ and contained 0.01% (v/v) Tween 80. $[CO] = 40 \mu M$, temperature $20^\circ C$, observation wavelength 415 nm . The enzyme concentrations were $1.29\text{--}1.69 \mu M$ for flash photolysis, and $0.62\text{--}0.76 \mu M$ after mixing for stopped-flow experiments.

pH	Buffer system	Rate after flash photolysis (s^{-1})	Stopped-flow rates	
			Faster process (s^{-1})	Slower process (s^{-1})
5	Sodium acetate	80.4	73.6	0.38
6	KH_2PO_4/Na_2HPO_4	60.3	62.7	0.46
7	KH_2PO_4/Na_2HPO_4	29.7	26.5	0.53
8	KH_2PO_4/Na_2HPO_4	28.3	—	—
8	Tris/HCl	24.9	—	—
9	Tris/HCl	21.2	—	—

($I = 0.05$). The pseudo-first-order rate, measured at 415 nm and at a constant concentration of CO ($40 \mu M$), changed little between pH 9 and pH 7, but was considerably more rapid at pH 6 and pH 5. Corresponding stopped-flow experiments were performed at pH 5, pH 6 and pH 7, and the rates of the faster process were found to match closely those seen after photolysis at the same pH (Table 2). The rate of the slower phase was also dependent upon pH, as it increased slightly with increasing pH. The faster process accounted for approx. 85% of the total kinetic absorbance change at 415 nm at both pH 5 and pH 6, and for approx. 90% at pH 7.

Kinetic difference spectra for the CO-binding and the CO-recombination reactions at pH 6 were constructed by measuring the amplitude of each kinetic process over a range of wavelengths close to the Soret region peak of CO-ferrocycytochrome *c*-551 peroxidase. In Fig. 3 these are compared with the static difference spectrum at pH 6 of CO-ferrocycytochrome *c*-551 peroxidase minus ferrocycytochrome *c*-551 peroxidase. The kinetic difference spectrum of the recombination (flash) reaction followed closely the static difference spectrum, and the faster stopped-flow process also showed the same spectral characteristics. The slower process, however, gave rise to a kinetic difference spectrum with both the positive and the negative peaks shifted to shorter wavelengths compared with the static difference spectrum. At 420 nm the two processes seen in the stopped-flow reaction were clearly giving rise to absorbance changes of opposite directions; the faster phase producing an increase in absorbance and the slower phase a decrease. However, in corresponding stopped-flow experiments at pH 5 the

Fig. 3. Comparison of static and kinetic difference spectra for CO-ferrocycytochrome *c*-551 peroxidase complex formation

The solid line represents the static difference spectrum, CO-enzyme complex minus reduced enzyme. Other points represent spectral changes observed in kinetic experiments: ●, after flash photolysis of CO-ferrocycytochrome *c*-551 peroxidase; ○, the faster process and Δ, the slower process observed during the reaction of dithionite-reduced enzyme and CO in the stopped-flow apparatus. The buffer was KH_2PO_4/Na_2HPO_4 , $I = 0.05$, pH 6, containing 0.01% Tween 80. $[CO] = 40 \mu M$, temperature $20^\circ C$. The enzyme concentration was $1.19 \mu M$ for static spectra and for flash photolysis measurements, and $0.76 \mu M$ after mixing for stopped-flow measurements.

slower process was found to cause only increases in absorbance in the spectral region examined (400–440 nm), with a maximal change close to 415 nm .

Discussion

The purification procedure described in this paper has been designed to take advantage of the most useful features of previously published methods. We consider that this results in a quicker, simpler method (only two types of gel and three columns are employed, and the use of acetone powders is avoided) of similar performance, in terms of yield

and purity, to the only other directly comparable procedures (Ellfolk & Soininen, 1970; Soininen, 1972). When $(\text{NH}_4)_2\text{SO}_4$ precipitations were used instead of the acid precipitation step, it was found that the enzyme suffered a progressive loss of a large part of its activity during subsequent purification. This may be a result of failure to prevent the co-purification of a proteinase enzyme with the peroxidase (Soininen & Ellfolk, 1975). The use of Sephadex CM-50 in 0.01M-sodium phosphate at pH 6 stems from the paper by Singh & Wharton (1973) on the purification of a protein which they called *Pseudomonas* 'cytochrome c-556' but which, on the basis of its size and of its amino acid analysis, was almost certainly the peroxidase. This gel has the useful property of binding the peroxidase tightly despite the high concentration of impurities present at an early stage in the preparation.

The instability of the enzyme during the course of degassing has not previously been reported. Loss of activity and of absorbance is accompanied by the appearance of flecks of red-brown precipitate on the surface of the solution. This poor solubility may be related to the fact that the enzyme is associated with the membrane fraction of the cell (Soininen *et al.*, 1970). A low concentration of detergent confers full stability. Enzyme protected by Tween 80 reacts with CO at the same rate as does the fraction of enzyme remaining after degassing in the absence of detergent, and Tween 80 also has no effect upon steady state assays of peroxidase activity. Therefore, it would seem that in most respects the protein is unaffected by the presence of detergent at the concentration used.

The results of the rapid-reaction studies on CO-ferrocycytochrome c-551 peroxidase appear to demonstrate the existence of a kinetic process common to both the formation of the complex from dithionite-reduced enzyme and CO, and the regeneration of the complex after photodissociation. The variation in rate of this reaction over a range of CO concentrations at pH 6, and over a range of pH values at a constant concentration of CO, was parallel in stopped-flow and flash photolytic techniques. The second-order rate constant at pH 6 was $1.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$.

We have restricted our detailed examination of the CO binding reaction to the pH range 5–9, because outside this range single exponentials are no longer observed and the results are not easily interpreted or compared. At pH 4 a protein sample prepared for a flash photolysis experiment under conditions comparable with those in Table 1 required about 3 h for full formation of the CO complex after addition of ligand. Despite this, kinetic observations of the sample did reveal recombination processes after photodissociation that were complete within 1 s. However, when the absorption spectrum of the

enzyme was run after a few flashes of light, it was found that the CO-bound species was no longer fully formed. Other spectrophotometric experiments we have carried out suggest that the stability of unliganded peroxidase is affected by dithionite at pH 4.

Although we did not observe any obvious spectral instability in corresponding experiments on reduced unliganded peroxidase at pH 10, the CO-binding behaviour was broadly similar to that at pH 4. Equilibration with added ligand was not 'immediate' and yet fast, multi-exponential processes were seen on flash photolysis. Absorption spectra run over a period of approx. 30 min after the sample had been exposed to a few flashes of light showed that some very slow reactions were also involved in CO recombination. Soininen & Ellfolk (1973a) have found evidence for the existence of a pH transition between neutral and alkaline forms of reduced peroxidase with a pK of 10. It is possible that at least some of the complexities described above may arise from this phenomenon. Nevertheless, examination of the data in Table 2 as a whole suggests that CO recombination with reduced enzyme does not appear to follow a pattern of behaviour equivalent to that found for the peroxidatic activity of the enzyme, after samples of oxidized enzyme had been incubated at different pH values (Soininen & Kalkinen, 1977). This earlier work showed that the catalytic properties of the enzyme were stable over a relatively narrow pH range, 6–7.4. In our experiments the CO reaction remained a single exponential over the pH range 5–9, with the rate constants indicating a transition in kinetic properties below pH 7, but showing little change between pH 7 and 9.

The slower process, observed only in the stopped-flow reaction, showed first-order kinetics. Since it did not occur after flash photolysis of the complex and there was a correspondence of the CO-dependent reactions seen in flow and flash experiments, it probably does not represent a conformational change after CO binding. Instead, it seems likely that two forms of the unliganded reduced enzyme exist in equilibrium, and that the slower stopped-flow process represents the conversion of a small proportion of the ferrocycytochrome c-551 peroxidase from a non-reactive state to a form which rapidly binds CO.

Thus a plausible mechanistic scheme for the CO-binding kinetics of ferrocycytochrome c-551 peroxidase may be formulated as follows, where A and A* are two spectrally distinct forms of reduced enzyme in slow equilibrium:

The process common to both flow and flash reactions is represented by the CO-binding reaction k_{+2} , whilst k_{+1} corresponds to the slow reaction observed by stopped-flow only. Upon photolytic dissociation of A-CO the amount of A* formed is negligible, since the equilibrium between the two unliganded forms is much slower than the CO recombination rate k_{+2} [CO] even at the lowest CO concentrations used. Hence no slower process is observed in the flash experiments.

The position of the equilibrium between the two unliganded forms did not change substantially over the pH range 5–7, although the rate of the slower stopped-flow phase showed a slight pH dependence, as did the spectral characteristics of the process. The present data do not allow a direct estimate of k_{-2} , the CO 'off' reaction, since the intercept of Fig. 2(b) must be very close to the origin. The CO titration curve at pH 6 published by Rönnberg & Ellfolk (1979) suggests that dithionite-reduced peroxidase has a very high affinity for CO, probably in excess of 10^7 M^{-1} (see Antonini & Brunori, 1971). This value, taken together with our present results, would mean that k_{-2} is somewhat less than 0.15 s^{-1} in the absence of a photolysing flash of light.

The proposed model is not without precedent; for instance, reduced *Pseudomonas* azurin has been shown to exist in two forms in equilibrium which display different activities toward electron acceptors (Brunori *et al.*, 1975). Further evidence of similar behaviour in the case of reduced *Pseudomonas* cytochrome *c*-551 peroxidase would of course be desirable, but we have not as yet found a suitable ligand other than CO.

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