Antibodies were also used to identify microsomal proteins modified after reaction with 4-maleimidotempo (Barber et al., 1984). Nitroxide-modified 'intact' microsomes subjected to separation followed by transfer to nitrocellulose revealed multiple bands indicating a broad pattern of protein substitution (Fig. 1). This could be further resolved by separation of the membrane proteins into integral and peripheral components by treatment with sodium carbonate (Fujiki et al., 1982). In addition, e.p.r. spectroscopy and densitometric analysis indicated preferential incorporation of spin-label into integral membrane proteins.

This work was supported by grants NIH S 507 RO5749, NSI PCM-8214001 and American Heart Association AG712.

Photolytic studies on cytochrome c peroxidase from Pseudomonas aeruginosa

COLIN GREENWOOD,* NICHOLAS FOOTE,* JIM PETERSON† and ANDREW THOMSON†
Schools of *Biological and †Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

A number of lines of investigation have clearly shown that the two covalently bound haem groups of the cytochrome c₅₅₅ peroxidase from Pseudomonas aeruginosa are inequivalent, optically, magnetically and in terms of their chemical reactivity: Aasa et al. (1981), Ellfolk et al. (1983) and Foote et al. (1983). Perhaps the most notable difference is the large difference between the redox potentials which are estimated to be +320 mV and -330 mV respectively. We have used a combination of low-temperature absorption and magnetic c.d. spectroscopy to study the nature of the photolyzed and thermally recombined forms of cytochrome c₅₅₅ peroxidase in different redox states.

Fig. 1 shows the near-i.r. magnetic c.d. spectra, recorded at 4.2 K and 4.9 T, of three enzyme species, namely half-reduced (a), fully reduced (b) and fully reduced carbon monoxide (c), before and after photolysis. Extensive changes in all the spectra occur on photolysis. The porphyrin-to-ferric iron charge-transfer band with a peak at 1450 nm, for example, is highly sensitive in the fully reduced state, and shoulder at 1250 nm which we identify with the low-potential haem. This high-spin (S = 2) state is stable indefinitely at 4.2 K but warming to a minimum of 20 K promotes a rapid ligand recombination. The photolabile ligand is most likely to be the methionine residue and our results, with other c-type cytochromes, suggest that there may be a series of cytochromes c with increasingly labile methionine ligands, i.e.

Horse heart < P. aeruginosa c₅₅₅ < P. aeruginosa cytochrome c peroxidase < Rhodospirillum rubrum c'

100% Low spin --- Photolability --- 100% High spin

Fig. 1. Near-i.r. magnetic c.d. spectra of derivatives of Pseudomonas aeruginosa cytochrome c₅₅₅ peroxidase showing the effect of photolysis

(a) Half-reduced enzyme (290 μM), containing 225 μM NADH and 12.5 μM phenazine methosulphate. (b) Fully reduced enzyme (200 μM), containing excess dithionite. (c) Fully-reduced CO-bound enzyme (110 μM), containing excess dithionite and 500 μM CO. Broken lines represent photolysed samples, and solid lines the same samples thermally recombined by warming to at least 50 K. Temperature 4.2 K; magnetic field 4.9 T.
spectrum of the low-potential haem persists after exposure to light. The low-potential haem in the ferrous state reacts rapidly with CO to form a low-spin CO adduct which can be broken down by light at room temperature. The anticipation was thus that in this derivative, both the high-potential haem and the low-potential carbon monoxide species would be photosensitive and at 4.2K would both be photolytically removed at room temperature we can be photolytically removed at room temperature we assume that it remains photolabile at 4.2K and that the rate of this reaction is about 1000 times faster at 4.2K than at 295K. Such a mechanism would prevent observation of a relatively long-lived, high-spin ferrous low-potential haem at 4.2K. This implies that quantum mechanical tunnelling of CO is relatively fast at 4.2K. Quantum mechanical tunnelling, in which a particle passes through a classically impenetrable barrier, has been observed in the recombination of CO with protohaem IX, and in the separated chains of haemoglobin and myoglobin (Alberding et al., 1976). The tunnelling rate depends upon the size of the barrier and upon the mass of the tunnelling particle whereas thermal recombination depends more on the height of the activation barrier. Our observation of rapid tunnelling at 4.2K and very fast thermal recombination at 293K are consistent with a low, narrow barrier towards recombination. This implies that very little constraint is placed upon the position of the proximal histidine group of the low-potential haem by the tertiary structure of the protein.

In this report we present evidence that nickel is a constituent of aerobic hydrogenase from aerobically grown Escherichia coli.

DAVID L. HALLAHAN and DAVID O. HALL
Department of Plant Sciences, Kings College, University of London, 68 Half Moon Lane, London SE24 9JF, U.K.

The hydrogenase from aerobically grown Escherichia coli has been purified and characterized as a dimer of identical subunits \( M_r 113,000 \), containing 12 iron and 12 acid-labile sulphur atoms per molecule (Adams & Hall, 1979). Recently, nickel has been reported as a constituent of a number of hydrogenases, including those produced during anaerobic growth of \( E. coli \) (S. P. Ballantine & D. H. Boxer, unpublished work). Crossed immunoelectrophoresis with antiserum to membranes from anaerobically grown cells shows that the precipitin arc of the enzyme fuses with that of isoenzyme 2 from anaerobic cells. This implies that the two enzymes are related (D. Boxer, personal communication).

In this report we present evidence that nickel is a constituent of active hydrogenase from aerobically grown \( E. coli \). Also, the deleterious effect of oxygen on the hydrogenase is investigated, using \(^{63}\text{Ni}\)- and \(^{59}\text{Fe}\)-labelled enzyme.

\( E. coli \) strain MRE 600 was obtained from the Centre for Applied Microbiology and Research, Porton Down, Wilts., U.K. For membrane preparation, bacteria were harvested after 18 hours growth on the batch medium of Elsworth et al. (1968), modified by addition of 56.6 ml of glycerol/l, 100 \( \mu \text{Ci of } ^{59}\text{FeCl}_2 (1.36 \text{nm}) \) and 100 \( \mu \text{Ci of } ^{60}\text{NiCl}_2 (0.143 \text{nm}) \). pH was adjusted to 6.8 before inoculation. Cell yield approximated 5.0 g/l, 63Ni uptake 2% and 59Fe uptake 75%.

Membranes were prepared from frozen cells by the method of Schnaitman (1981), with three cycles of freezing and thawing in the presence of lysozyme and EDTA. The buffer used was 20 mM-Tris/Cl, pH 8.0.

For octyl-Sepharose chromatography, 10 ml of membranes were solubilized with 3% (w/v) deoxycholate, \((\text{NH}_4)_2\text{SO}_4\) added to 25% saturation, and the mixture centrifuged at 7000g for 20 min. The supernatant was loaded onto a column \((1.2 \text{cm} \times 12.0 \text{cm}) \) of octyl-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.1 M-(\(\text{NH}_4\))2SO4 at 60 ml/h. After washing with buffer alone, hydrogenase was eluted with a 100 ml linear gradient of 0-3% (w/v) deoxycholate.

Samples for polyacrylamide-gel electrophoresis were mixed 1:1 with 6% (w/v) deoxycholate and incubated with shaking at 30\(^\circ\)C for 1 h. Electrophoresis was conducted in air at 4\(^\circ\)C on 8% gel rods in a non-denaturing system (Neville, 1971). Gels were stained for hydrogenase activity (Adams & Hall, 1979), then quickly frozen and cut into slices (2 mm). Slices were dried, then solubilized with 200 \( \mu \text{l} \) of 30% (w/v) hydrogen peroxide at 90\(^\circ\)C. Radioactivity was located by dual-label liquid scintillation counting. Duplicate gels were subjected to prolonged staining to ensure no additional bands appeared (Adams & Hall, 1979).

Hydrogenase was assayed by measuring hydrogen evolution from reduced Methyl Viologen (Adams & Hall, 1979). Protein was determined by the method of Lowry et al. (1951), with fat-free bovine serum albumin as standard.

Membranes prepared from \( E. coli \) grown on \(^{63}\text{Ni}\) and \(^{59}\text{Fe}\) were solubilized and the extract loaded onto a column of octyl-Sepharose. Seventy percent of the hydrogenase activity loaded was eluted with 0.6% (w/v) deoxycholate, coincident with a band of radioactivity containing \(^{63}\text{Ni}\) and \(^{59}\text{Fe}\). The peak fraction was found to have a hydrogenase specific activity of 24.0 units/mg of protein, and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed eight protein bands. The peak fraction was used directly for non-denaturing gel electrophoresis.

The resulting gel profile (Fig. 1a) shows an aggregate of \(^{63}\text{Ni}\) and \(^{59}\text{Fe}\) proteins which do not migrate into the gel, but the main band of \(^{63}\text{Ni}\) and \(^{59}\text{Fe}\) clearly corresponds to hydrogenase, with an \( R_f \) of 0.24.

The effect of oxygen on the metal content of the enzyme was investigated by exposing the preparation used above to air at 30\(^\circ\)C for 24 h in the presence of 3% (w/v) deoxycholate. This resulted in a 55% loss of activity. When subjected to electrophoresis as before, the gel profile of Fig. 1(b) was obtained. A major \(^{63}\text{Ni}\) and \(^{59}\text{Fe}\) band migrated to an \( R_f \) of 0.24, representing active hydrogenase. However, though the \(^{59}\text{Fe}\) content of the enzyme seems largely unaffected by exposure to air, the \(^{63}\text{Ni}\) band was