A Comparative Survey of the Iron–Sulphur Centres in the Cytoplasmic Membranes of *Pseudomonas cichorii* and *Pseudomonas aptata*

By DAVIDE ZANNONI¹ AND W. JOHN INGLEDEW²*

¹ Institute of Botany, University of Bologna, Bologna, Italy

² Department of Biochemistry and Microbiology, University of St Andrews, St Andrews, UK

(Received 2 December 1983; revised 6 February 1984)

The respiratory chains of the related phytopathogenic bacteria *Pseudomonas cichorii* and *Pseudomonas aptata* were investigated using the technique of electron paramagnetic resonance which readily detects iron–sulphur centres. In the cytoplasmic membranes of both bacteria three ferredoxin-like centres and a centre detected in the oxidized state ($g = 2.015$) were resolved. In addition, a Rieske-type iron–sulphur centre ($g_y = 1.90$) was observed in *P. cichorii* but not in *P. aptata*. The absence of the Rieske centre in *P. aptata* parallels the absence of cytochromes *c* in this bacterium.

INTRODUCTION

Pseudomonas cichorii and *Pseudomonas aptata* are fluorescent phytopathogenic bacteria. A study of their membrane-bound cytochromes has revealed that a linear respiratory chain is functional in *P. aptata*, cytochromes *c* being absent and the terminal oxidase being an auto-oxidizable cytochrome *b* (called cytochrome *o*). In contrast, *P. cichorii* appears to retain a similar cytochrome *o* pathway but has in addition a second pathway involving cytochromes *c* to a distinct auto-oxidizable cytochrome *b*. These cytochromes *b* differ in their mid-point oxidation–reduction potentials and their sensitivities to cyanide (Zannoni, 1982).

In this paper we have applied the techniques of electron paramagnetic resonance spectroscopy (EPR) to the study of these respiratory chains.

METHODS

Cell growth, harvesting, cell breakage and the preparation of cytoplasmic membrane fragments. *Pseudomonas cichorii* (NCPPB 907) and *P. aptata* (NCPPB 2664) were grown in a medium of the following composition: D-glucose, 20 g; yeast extract (Difco), 5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; KH_2PO_4 , 0.6 g; K_2HPO_4 , 1 g. The medium was adjusted to pH 7.2 in 1 litre of distilled water. Growth medium (10 l) supplemented with 0.5 ml antifoam C emulsion was inoculated with 200 ml of a growing culture (initial absorbance 0.05 at 660 nm). The cells were harvested after growth for 18 h (final absorbance of about 1.2) at 30 °C in a Microferm Fermentor (New Brunswick Scientific Co.), and then stirred at 250 r.p.m. and bubbled with humidified air at 6 l min⁻¹. The harvested cells were broken by French pressure cell treatment and the membrane fraction was separated by differential centrifugation as previously described (Zannoni, 1982).

Electron paramagnetic resonance spectroscopy. EPR spectra were obtained using a Bruker 200 D EPR spectrometer (Bruker Analyt. Messtechnik, Forchleim, FRG) equipped with a cryostat (Oxford Instruments, Oxford, UK) and a liquid helium transfer system. The temperature was measured with a thermocouple.

Determination of oxidation–reduction mid-point potentials and other assays. Oxidation–reduction titrations were performed as described by Dutton (1971). The membrane preparation was suspended, to 20 mg protein ml⁻¹, in 50 mM-TES buffer pH 7.0. The following mediators were used at between 20 and 50 μM : dibromophenol indophenol; 2-hydroxy-1,4-naphthoquinone; 1,4-naphthoquinone; duroquinone; indigo-disulphonate; indigo-tetra-sulphonate; 2,3,5,6-tetramethylphenylenediamine; *N*-methylphenazonium methosulphonate; methylviologen;

Abbreviation: HiPIP, high potential iron protein.

benzylviologen; phenosafranine; anthraquinone-2-sulphonate; 2-hydroxy-naphthoquinone. The samples were stored in liquid nitrogen until use. Protein concentrations were estimated by a modification of the Lowry method that included SDS.

RESULTS

In this paper the term ferredoxin is used to indicate an iron-sulphur centre which is EPR-detectable in its reduced form. The term HiPIP (high potential iron protein) is used for a centre which is EPR-detectable in its oxidized form. The EPR spectra shown are the first derivatives of the absorption spectra. This is standard practice with EPR and it gives much better resolution than the absorbance spectrum. A ferredoxin has either rhombic or axial geometry: the former will lead to a three-line first-derivative spectrum (g_z peak, g_y peak and trough, g_x trough) and the latter to a two-line spectrum ($g_{\parallel}$ peak, $g_{\perp}$ peak and trough, i.e. g_x and g_y are superimposed). Each iron-sulphur centre will have its own three (or two) characteristic g values [calculated from $g = v/\beta H$, where β is a constant (the Bohr magneton), v the microwave frequency and H the magnetic field strength at the position of the line]. Spectra were taken at different temperatures because each centre has its own optimum temperature for observation (determined by its mechanisms and rates of relaxation and also by the incident microwave power) above and below which the signal intensity lessens (broadening and power saturation, respectively).

Electron paramagnetic resonance spectra of P. aptata

EPR spectra of *P. aptata* membranes either oxidized, NADH-reduced (anaerobic) or dithionite-reduced, are shown in Fig. 1 and the corresponding spectra of *P. cichorii* are shown in Fig. 2. In the spectrum of dithionite-reduced membranes obtained at 8 K, two centres, distinguishable by their g values and spectral lines shapes, could be resolved. Monitoring the various peaks and troughs from redox titrations and over a range of temperatures (not shown) led to the assignment of the $g = 2.046$ peak and the $g = 1.89$ trough to the same centre as g_z and g_{xy} , respectively (some rhombic distortion is suggested by the lineshape around $g = 1.89$). The line shape of this centre has relatively little spectral overlap from other centres in the NADH-reduced sample (Fig. 1*a*). In the NADH-reduced 8 K spectrum there was a centre with a g_z at 2.02 and a g_{xy} in the region of 1.93, but not so large as in the dithionite-reduced spectrum.

When spectra obtained at the higher temperature of 22 K were examined (Fig. 1*b*) it was observed that: (a) the $g = 2.046/1.89$ centre was not present; (b) the rhombicity of the $g = 2.02/1.93$ centre could be seen as it was not free from overlap with the $g = 2.046/1.89$ centre; (c) the line shape around the $g = 1.93$ centre was different when comparing the dithionite-reduced sample with the NADH-reduced sample and (d) the ratio of the signal amplitudes of the NADH and dithionite samples at $g = 1.93$ was very different at 22 K compared to 8 K. Points (c) and (d) suggest the presence of two $g = 2.01/1.93$ centres, one being substrate-reducible (NADH), best observed at the higher temperatures and heavily saturated at 8 K (microwave power in Fig. 1 is 22 mW), and the other not being NADH-reducible but dithionite-reducible and best observed at low temperatures. Studies of temperature dependence of the respective spectra and the analysis of redox titrations (Fig. 3), which resolved the $g = 2.02/1.93$ centre into two components, confirmed this suggestion. Behaviour such as this, with iron-sulphur centres of this type, is well documented in succinate dehydrogenases and fumarate reductases (see Discussion).

In the oxidized sample at 8 K a large EPR signal with a peak at $g = 2.015$ was observed. This centre was similar to centres described in succinate dehydrogenases, fumarate reductases and some other enzymes such as aconitase, and was due to a four or three iron sulphide cluster which is paramagnetic (EPR-detectable) in its physiologically oxidized form (designated HiPIP). Quantification of this HiPIP centre indicated that it was present at a similar concentration to the $g = 2.02/1.93$ centres, which are also typical of succinate dehydrogenase. Measured under non-saturating conditions, HiPIP gave $0.13 \text{ nmol (mg protein)}^{-1}$; the $g = 2.02/1.93$ dithionite-

Fig. 1

Fig. 2

Fig. 1. Electron paramagnetic resonance spectra of cytoplasmic membranes of *P. aptata*. Membrane samples were dithionite-reduced, NADH-reduced or oxidized. Spectra were taken at (a) 8 K and (b) 22 K. The numbers at the bottom of the figures are g values. The concentration of proteins was 40 mg ml^{-1} . The EPR conditions were: microwave power, 22 mW; modulation amplitude, 10^{-3} tesla; microwave frequency, 9.49 GHz; modulation frequency, 100 KHz.

Fig. 2. Electron paramagnetic resonance spectra of cytoplasmic membranes of *P. cichorii*. Spectra were taken at (a) 8 K and (b) 22 K. Membrane samples were dithionite-reduced, NADH-reduced or oxidized. The numbers at the bottom of the figures are g values. Other conditions were as for Fig. 1.

reduced centres gave $0.15 \text{ nmol (mg protein)}^{-1}$ and the NADH-reduced centres gave $0.13 \text{ nmol (mg protein)}^{-1}$; and the $g = 2.046/1.89$ centre gave $0.06 \text{ nmol (mg protein)}^{-1}$.

Oxidation-reduction titrations were performed to determine mid-point potentials (Fig. 3). The HiPIP centre gave a mid-point potential (pH 7.0) of approximately +100 mV and the $g = 2.046/1.89$ ferredoxin a potential of -210 mV. The $g = 2.02/1.93$ ferredoxin titrated biphasically, suggesting two components (but see Discussion) with mid-point potentials at -60 mV and -230 mV.

EPR spectra of *P. cichorii*

Figure 2 shows EPR spectra of oxidized and dithionite- or NADH-reduced *P. cichorii* membranes. The spectra were in some respects quite similar to those of *P. aptata*. The $g = 2.046/1.89$ centre was present at the same concentration as in *P. aptata* at $0.06 \text{ nmol (mg protein)}^{-1}$, although the $g = 2.02/1.93$ centres (again resolvable into two components) were present at a higher concentration [dithionite-reduced, $0.38 \text{ nmol (mg protein)}^{-1}$]. In *P. cichorii* membranes an additional centre characterized by a $g_y = 1.90$ was seen at 22 K. This resonance was similar to those of the Rieske iron-sulphur centres observed in other respiratory and photosynthetic electron transport chains in terms of its mid-point potential (+200 mV, Fig. 3), g values and temperature dependence (Rieske *et al.*, 1964). This Rieske-like centre was clearly distinguishable from the $g = 2.046/1.89$ centre on the basis of these same criteria. The Rieske-like centre also had a g_x resonance weakly indicated at $g = 1.78$; the expected g_z signal at $g = 2.03$ could not be resolved because of overlapping signals.

Fig. 3. Analysis of oxidation-reduction titrations of membrane fragments of (a) *P. aptata* and (b) *P. cichorii*. The curves are drawn for $n = 1$ oxidation-reduction reactions. The EPR conditions were as for Fig. 1. The titration of the $g = 1.93$ signal is shown from spectra taken at 8 K (■) and 22 K (▲). The $g = 1.89$ data (○) are from 8 K spectra and are shown only for *P. aptata* (data are the same for *P. cichorii* but not shown). The Rieske data (□) were obtained from 22 K spectra of *P. cichorii*. The $g = 2.015$ data (●) are from spectra at 8 K.

DISCUSSION

Pseudomonas aptata and *P. cichorii* membranes contain a $g = 2.046/1.89$ ferredoxin with a mid-point potential of approximately -210 mV (Fig. 3, data not shown for *P. cichorii*), two $g = 2.02/1.93$ centres distinguishable by temperature dependence and mid-point potential (-60 mV and -230 mV for *P. aptata* and -50 mV and -260 mV for *P. cichorii*), and a HiPIP-type centre. A Rieske-type ferredoxin centre is present in *P. cichorii* but not in *P. aptata*.

Three of the iron-sulphur centres in each organism could be tentatively assigned to their succinate dehydrogenases by analogy with other systems. All known succinate dehydrogenases contain two $g = 1.93$ centres and a HiPIP centre with mid-point potentials close to those reported here (Ohnishi *et al.*, 1976; Ingledew & Prince, 1977; Malkin & Bearden, 1978; Ingledew *et al.*, 1980; Beinert & Albracht, 1982). An additional similarity between these centres and those of known succinate dehydrogenases is the difference in temperature dependence between the substrate-reducible $g = 1.93$ centre (the higher potential centre) and the dithionite-reducible centre. Although in this paper the data are interpreted as indicating the presence of two $g = 2.02/1.93$ ferredoxins, a more complex explanation exists in which only one

centre is present. A change in the relaxation rate of the centre (hence temperature dependence) through titration of an adjacent but unknown species can give the appearance of two centres. However, this argument is beyond the scope of this paper (see Beinert & Albracht, 1982).

The signal observed at $g = 1.90$ at higher temperatures (22 K and above) and only found in *P. cichorii* is taken to be a Rieske-type centre by analogy with the centre detected in the ubiquinol-cytochrome *c* complex resolved from eukaryotic mitochondria by Rieske (Rieske *et al.*, 1964). The g_y resonance is seen at $g = 1.90$, the g_x resonance is weakly indicated at $g = 1.78$ and the g_z resonance is expected at 2.03 but cannot be distinguished because of overlapping signals of unknown origin. The correlation between the presence of cytochromes *c* in *P. cichorii* and the Rieske centre, compared to their absence in the closely related *P. aptata*, is of interest and suggests that in these pseudomonads cytochromes of the *c* type and the Rieske centre are phenotypically and/or genetically linked. Such a correlation has been suggested before, but from comparison of widely different cell types (e.g. *Escherichia coli* and *Paracoccus denitrificans*; Ingledew *et al.*, 1980). The observations reported in this paper strengthen the support for a combined function of cytochromes *c* and Rieske centres.

D.Z. was the recipient of a short-term fellowship from the European Molecular Biology Organization. The support of the Science and Engineering Research Council of the UK is gratefully acknowledged.

REFERENCES

- BEINERT, H. & ALBRACHT, S. P. J. (1982). New insights, ideas and unanswered questions concerning iron-sulphur clusters in mitochondria. *Biochimica et biophysica acta* **683**, 245–277.
- DUTTON, P. L. (1971). Oxidation-reduction potential dependence of the interaction of cytochromes, bacteriochlorophyll and carotenoids at 77K in chromatophores of *Chromatium D* and *Rhodopseudomonas aeruginosa*. *Biochimica et biophysica acta* **226**, 63–80.
- INGLEDEW, W. J. & PRINCE, R. C. (1977). Thermodynamic resolution of the iron sulphur centres of the succinic dehydrogenase of *Rhodopseudomonas sphaeroides*. *Archives of Biochemistry and Biophysics* **178**, 303–307.
- INGLEDEW, W. J., REID, G. A., POOLE, R. K., BLUM, H. & OHNISHI, T. (1980). The iron sulphur centres of aerobically grown *Escherichia coli* K12. An electron paramagnetic resonance study. *FEBS Letters* **111**, 223–227.
- MALKIN, R. & BEARDEN, A. J. (1978). Membrane bound iron sulphur centres in photosynthetic systems. *Biochimica et biophysica acta* **505**, 147–181.
- OHNISHI, T., SALERNO, J. C., WINTER, D. B., LIM, J., YU, C. A., YU, L. & KING, T. E. (1976). Thermodynamic and EPR characteristics of two ferredoxin-type iron-sulphur centres in the succinate-ubiquinone reductase segment of respiratory chain. *Journal of Biological Chemistry* **251**, 2094–2104.
- RIESKE, J. S., ZAUG, W. S. & HANSEN, R. E. (1964). Studies on the electron transfer system, LIX. Distribution of iron and of the component giving an electron paramagnetic resonance signal at $g = 1.90$ in subfractions of complex III. *Journal of Biological Chemistry* **239**, 3023–3036.
- ZANNONI, D. (1982). Cytochrome *b* type oxidases in the respiratory chains of the phytopathogenic fluorescent bacteria *Pseudomonas cichorii* and *Pseudomonas aptata*. *Archives of Microbiology* **133**, 267–274.