Redox potential of the cytochrome *c* in the flavocytochrome *p*-cresol methylhydroxylase

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The redox potential of the cytochrome *c* in 5 flavocytochrome *c* proteins, all *p*-cresol methylhydroxylases purified from species of *Pseudomonas*, was measured. All gave similar values ranging from 226–250 mV. Two of the enzymes, from *Pseudomonas putida* NC1B 9866 and NC1B 9869, were resolved into their flavoprotein and cytochrome subunits and the redox potentials of the isolated cytochrome *c* subunits measured. The values for these were 60–70 mV below those for the whole enzymes but, in both cases, reconstitution of active enzyme by addition of the flavoprotein subunit restored the original potential.

p-Cresol methylhydroxylase *Flavocytochrome* *Cytochrome c* *Redox potential* *Reconstitution*

1. INTRODUCTION

Flavocytochrome *c* is one small class of *c*-type cytochromes where the cytochrome is associated with a flavoprotein subunit [1]. They were first isolated from phototrophic sulphur bacteria where they are involved in sulphur metabolism but, more recently, they have been found in species of *Pseudomonas* where they serve as an enzyme of the *p*-cresol degradative pathway, hydroxylating *p*-cresol to give *p*-hydroxybenzyl-alcohol [2,3]. Besides their different functions there are marked differences in the subunit size and composition of the flavocytochromes from the two groups and it is here shown that the redox potentials of the haem also differ, those of the *p*-cresol methylhydroxylases being considerably higher than the average of +55 mV reported for the haem in the flavocytochromes from the phototrophic sulphur bacteria [1]. The flavocytochrome from *Pseudomonas putida* (NCIB 9869) consists of two subunits of equal M_r (57000) and recently authors in [4] described its resolution into flavoprotein and cytochrome *c* subunits which, when mixed, reassociated to give active enzyme. The redox potentials of the resolved cytochrome *c* subunits from two *p*-cresol methylhydroxylases have now been measured. These were lower than for the unresolved enzyme but, on reconstitution with the flavin subunits, the original redox potentials were restored.

2. MATERIALS AND METHODS

The organisms were grown and enzymes from *P. putida* strains NCIB 9866 and 9869 purified as described previously [2,3]. Similar methods were used for the purification of *p*-cresol methylhydroxylases from *P. alcaligenes* (NCIB 9867) and *P. testosteroni* (NCIB 8955) (unpublished). Purified enzymes were dialysed against distilled water, lyophilized and stored at −20°C.

Subunits of flavocytochromes were separated by preparative isoelectric focusing as in [4] using an LKB 2117 multiphor apparatus. In each case 30 mg of purified enzyme were applied to the gel and, after electrophoresis for 6 h, the separated yellow and red bands were removed and the proteins eluted from the gel with 50 mM phosphate buffer, pH 7.0. The cytochrome fraction was dialysed against distilled water, lyophilized and stored at −20°C. The flavoprotein was dialysed against 50 mM phosphate buffer, pH 7.0, and stored at −20°C.

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Redox potentials were determined by potentiometric titration with dithionite and potassium ferricyanide using a stirred anaerobic cuvette of similar design to that of Dutton [5]. Enzyme (~2 mg) was dissolved in 50 mM Mops (4-morpholinepropanesulphonic acid)–NaOH buffer (pH 7.0) containing 50 mM KCl. The following mediators were added: 50 μM diaminodurene, 30 μM phenazonium methosulphate and 30 μM phenazonium ethosulphate. Anaerobic conditions were maintained by a flow of argon gas purged of traces of oxygen by passage through Fieser's solution. The cell potential at ambient temperature was measured with a platinum electrode by reference to a calomel electrode connected by a salt bridge to the cuvette contents. The degree of reduction of the cytochrome was monitored spectrophotometrically using a Hitachi-Perkin-Elmer dual wavelength spectrophotometer (model 356). For whole enzymes the absorbance change at 550 nm was followed relative to a reference wavelength of 540 nm. For isolated subunits the corresponding wavelengths were 552 nm and 542 nm.

3. RESULTS AND DISCUSSION

p-Cresol methylhydroxylases were purified from several species of *Pseudomonas* in which metabolism of *p*-cresol is initiated by oxidation of the methyl group to carboxyl. Three of the proteins from *P. putida* have been described in [2,3] and now similar flavocytochrome *c* enzymes have also been isolated from *P. alcaligenes* and *P. testosteroni*. Redox potentials for the cytochrome *c* in each enzyme were determined from plots of potentiometric titrations and are given in table 1. In each case the plot was that for a single electron transfer. All give quite similar values, between 220 mV and 250 mV, much higher than those of the only other known flavocytochromes *c* of the phototrophic sulphur bacteria which range from 8–29 mV for the *Chromatium* protein to 98 mV for that of *Chlorobium* [1]. This presumably reflects the different physiological roles of the proteins in the two groups and clearly, with differences also in subunit size and composition, there is considerable diversity within this one small class of *c*-type cytochromes.

Authors in [4] reported the resolution of one of the *p*-cresol methylhydroxylases (hydroxylase A from *P. putida* NCIB 9869) into its cytochrome *c* and flavoprotein subunits which, when mixed, reassociated to give active enzyme. Their separation procedure was used to obtain cytochrome *c* subunits from both the hydroxylase A and the enzyme of *P. putida* NCIB 9866. Redox potentials of these subunits were measured (table 2) and in both cases values were about 60–70 mV below those for whole enzymes. A plot of the results of a titration for cytochrome *c* from *P. putida* NCIB 9866 is shown in fig.1 and corresponds to a one electron transfer. After the titration an excess of the flavin subunit was added to reconstitute the *p*-cresol methylhydroxylase and the cytochrome retitrated. This now gave a value for its redox potential which was about the same as for unresolved enzyme (fig.1, table 2). Similarly the redox potential of the cytochrome subunit of hydroxylase A from *P.*

Table 1

Redox potentials of the cytochrome *c* in *p*-cresol methylhydroxylases from various organisms

Bacterium	$E_{m,7}$ (mV)
P. putida NCIB 9866	+250
P. putida NCIB 9869	
Hydroxylase A	+248
Hydroxylase B	+230
P. alcaligenes NCIB 9867	+250
P. testosteroni NCIB 8955	+226

Table 2

Redox potentials of isolated cytochrome *c* subunits and reconstituted *p*-cresol methylhydroxylases

Bacterium	$E_{m,7}$ (mV)
P. putida NCIB 9866	
Cytochrome *c* subunit	+180
Reconstituted enzyme	+247
P. putida NCIB 9869 (hydroxylase A)	
Cytochrome *c* subunit	+187
Reconstituted enzyme	+249

Enzymes were reconstituted by adding about a 2-fold excess of the flavoprotein subunit to a solution containing the cytochrome *c* subunit

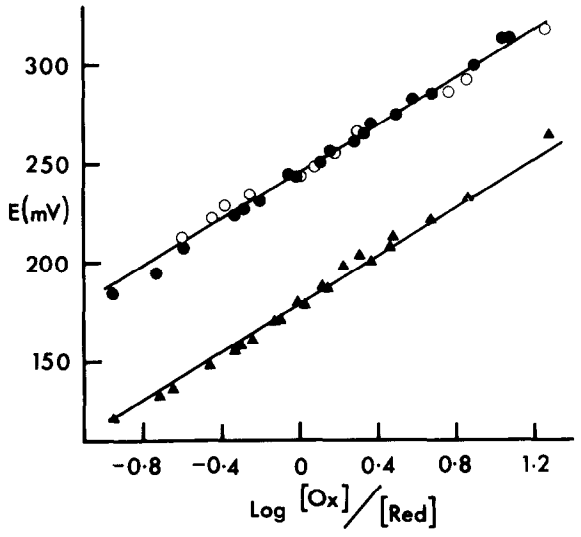

Fig.1. Redox titrations of the cytochrome *c* of *p*-cresol methylhydroxylase from *P. putida* NCIB 9866. Conditions were as in section 2: (●) titration of purified enzyme; (▲) titration of isolated cytochrome *c* subunit (0.5 mg) alone and (○) after addition of the flavoprotein subunit (1 mg).

putida NCIB 9869 returned to that of unresolved enzyme when an excess of flavin subunit was added. It has been shown that redox potentials of *c*-type cytochromes can be influenced by binding with other materials, particularly membranes. For example, the solubilization of a membrane-bound cytochrome *c* from *Azotobacter* lowered its redox potential from 320 mV to 278 mV [6] and shifts in E_m of mammalian cytochrome *c* from 280 mV to 230 mV when bound to various membranes have been shown [7]. Properties of haem groups in proteins may be influenced by the ligands to the iron and the environment provided by the folded polypeptide [8]. It has been suggested that in cytochrome *c* the length and orientation of the iron–methionine bond is important in determining redox potentials and this property may, therefore, be influenced by any change in protein structure which alters the length or orientation of this bond [9]. Thus the shift in redox potential of the cytochrome *c* subunit of *p*-cresol methylhydroxylase that occurs on mixing with the flavin subunit possibly reflects changes in structure due to interactions of the subunits to give the reconstituted enzyme. These interactions are reported to affect also the flavoprotein subunit in that combination with the cytochrome may increase the rate of electron transfer between substrate and covalently-bound flavin [4].

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