CYTOCHROME *c* LINKED NICOTINIC ACID HYDROXYLASE IN *PSEUDOMONAS OVALIS* CHESTER

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1. Introduction

An inducible membrane bound system for the oxidation of nicotinic acid occurs in some *Pseudomonas* species [1,2]. Previous work with *Pseudomonas ovalis* Chester had indicated that the enzyme, nicotinic acid hydroxylase, might be coupled to an electron transport system at the level of cytochrome c_{551} [3]. The solubilisation, by sodium deoxycholate, of the hydroxylase and associated cytochromes has been achieved [4]. In this paper the properties of the detergent solubilised hydroxylase are described and its similarities with other membrane-bound, cytochrome linked enzymes is discussed.

2. Materials and methods

Growth and preparation of the cell wall/membrane fraction from *Ps. ovalis* Chester have been described [3]. Nicotinic acid hydroxylase was prepared by the method of Hunt [4] with the following modification. Prior to the final dialysis stage, the preparation was incubated with 100 μg/ml, wheat germ lipase (Sigma Chemicals, London) for 1 hr at 30°. After dialysis for 16 hr the preparation was centrifuged at 100,000 *g* for 1 hr. The supernatant was used as the partially purified nicotinic acid hydroxylase.

Nicotinic acid hydroxylase was assayed spectrophotometrically, using 2,6-dichlorophenol indolphenol (DICIP) as an acceptor, by following the decrease in absorbance at 600 nm. The reaction mixture contained, in 3 ml total volume, 150 μmoles K-phosphate (pH 6.2), 90 μg DICIP, 30 μmoles Na-nicotinate, and 100–300 μg enzyme protein. Assays and spectra were done at room temp. on a Pye Unicam SP 1800 recording spectrophotometer.

3. Results

After dialysis, 85–90% of the deoxycholate solubilised hydroxylase activity was found in the pale straw coloured supernatant. The remainder of the activity was associated with the dark red-brown pellet which contained the bulk of the "solubilised" cytochromes. If the lipase treatment was omitted considerably more hydroxylase activity was associated with the insoluble material (up to 40%).

Four separate preparations of the hydroxylase had an average activity of 1096 μmoles DICIP reduced/min/mg protein, representing a 46 × increase in activity over the wall/membrane fraction. The preparation did not show any nicotinic acid oxidase activity nor would it couple the reduction of DICIP to other substrates such as succinate or NADH. The pH optimum for DICIP reduction was found to be 6.2 (fig. 1) and the K_m for nicotinate was determined to be 2×10^{-5} M.

Potassium ferricyanide and phenazine methosulphate were also readily reduced by the enzyme in the presence of nicotinate but methylene blue and *Pseudomonas* azurin were poor acceptors. The pH optimum for ferricyanide reduction was found to differ from that obtained with DICIP, and to occur at pH 7.0 (fig. 1). The reduction of mammalian cytochrome *c* was found to be a variable property. One preparation reduced it at about half the rate of DICIP

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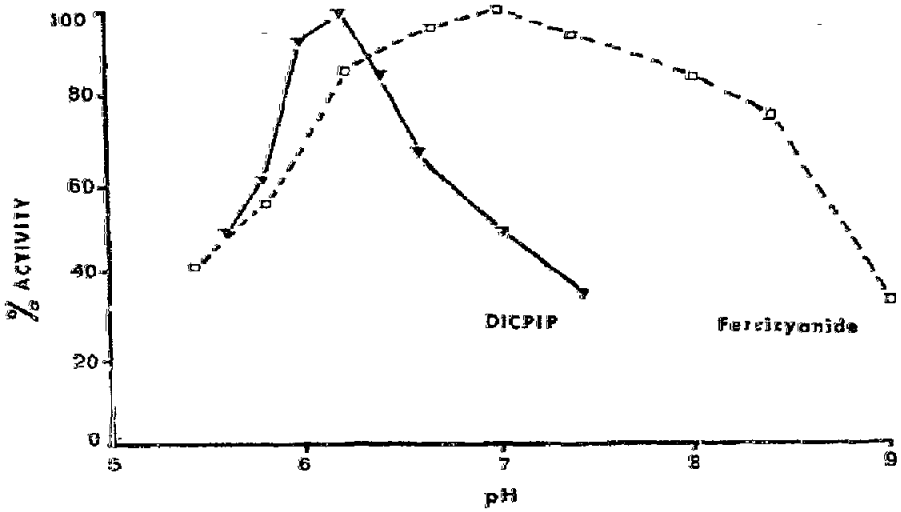

Fig. 1. pH optima for ferricyanide and DICPIP-linked nicotinic acid hydroxylase activity. Results are expressed as % of rate at optimum pH. Tris-maleate (50 mM) was used for pH's up to 7.4 and Tris-HCl (50 mM) for pH's 7.4–9.0. No difference in activity was found in Tris or phosphate buffers at the same pH.

reduction but in others very much lower rates were observed.

In the absence of nicotinate, the hydroxylase has a single absorption maximum in the visible region at 410 nm. Reduction of the enzyme results in the shifting of this maximum and the appearance of additional peaks characteristic of cytochromes. Fig. 2a shows a difference spectrum obtained with nicotinate as the reductant. Maxima occur at 418, 521 and 550 nm, typical of cytochrome *c*, but there is practically no bleaching in the flavin region of the spectrum. With dithionite as reductant the Soret maximum is shifted to 428 nm and additional shoulders occur at 530 and 559 nm (fig. 2b). Carbon monoxide difference spectrum (fig. 2c) indicates the presence of considerable cytochrome *o* which probably accounts for the absorbance at 559 and 530 nm [5]. Only 12% of the cytochrome *o* was found to be reducible by nicotinate.

Neither FMN or FAD had any affect on the enzyme activity as measured by DICIP reduction. Incubation with acriflavine was not inhibitory (table 1). Precipitation by ammonium sulphate at acid pH [6] resulted in considerable loss of activity but this could not be restored by either flavin nucleotide. This together with the lack of bleaching in the flavin region of the difference spectrum by nicotinate suggests that the enzyme is not a flavoprotein nor linked to the cytochrome system through a flavin. When the enzyme was treated with *o*-phenanthroline a reddish complex, with an absorption maximum at 510 nm, was formed. This has been reported to be characteristic of non-haeme iron [7]. Addition of nicotinate intensified the colour (fig. 3). Dialysis against 1 mM *o*-phenanthroline for 6 hr followed by dialysis against K-phosphate buffer (50 mM, pH 7.0) for 6 hr caused over 90% loss of activity but this could not be re-

Table 1
Effects of inhibitors and chelating agents on enzyme activity.

Inhibitor	Concentration (mM)	Inhibition (%)
8-Hydroxyquinoline	5	77
Dipyridyl	5	76
Hydroxylamine	5	22
EDTA	5	+18*
Cu^{2+}	0.1	88
p-Hydroxy mercuribenzoate	1	92
Acriflavine	1	0

The enzyme, 1 mg/ml in 50 mM Tris-HCl buffer pH 7.4, was incubated at 4° for 2 hr with the chelating agents and acriflavine at the concentrations indicated above. The sulphydryl reagents were added immediately before assay. * The result obtained with EDTA indicates a stimulation of enzyme activity, all other results indicate % inhibition compared with a control, without addition, that was incubated for the same period.

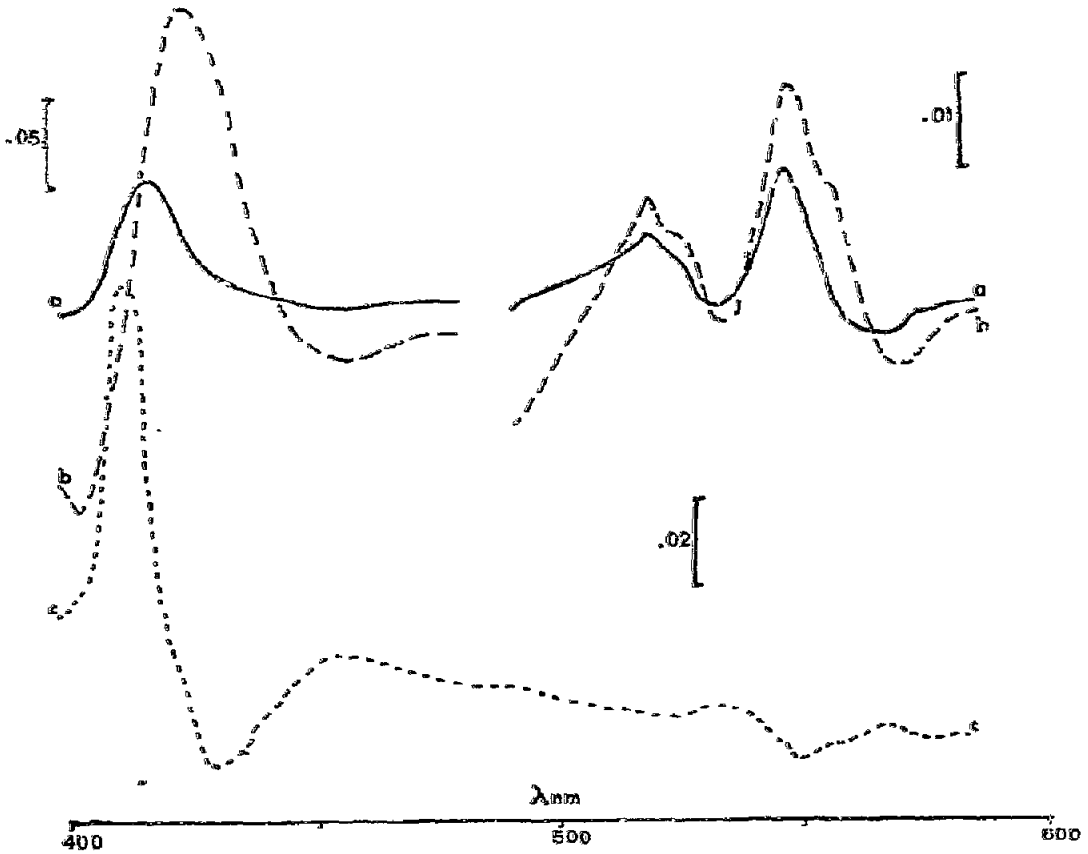

Fig. 2. Spectral properties of the nicotinic acid hydroxylase preparation. Curve a (——) Oxidised minus nicotinate reduced difference spectrum. Curve b (– – –) Oxidised minus dithionite reduced difference spectrum. Curve c (- - -) Carbon monoxide difference spectrum. Cuvettes (1 cm light path) contained either 8 mg/ml protein (curves a and b) or 4 mg/ml protein (curve c).

stored by subsequent incubation with a variety of metal ions. Other chelating agents were also inhibitory (table 1), as were sulphydryl reagents, but EDTA stimulated slightly and helped to prevent gradual loss of enzyme activity when the enzyme was stored at room temp.

4. Discussion

The dehydrogenases for NADH, succinate and malate in a wide variety of microorganisms are flavoproteins coupled to the electron transport system through a quinone and cytochrome *b* [8]. The malate dehydrogenase in *Ps. ovalis* has been shown to be of this type [9]. The results presented here indicate that the inducible nicotinic acid hydroxylase is a different class of respiratory enzyme. Spectral evidence from the partially purified enzyme suggest that it is linked to cytochrome *c* and supports similar conclusions obtained with cell wall/membrane fractions [3]. There is no evidence that the enzyme is a flavoprotein, but the involvement of non-haeme iron is possible.

Two other inducible respiratory enzymes in *Ps. putida*, L-pipecolate dehydrogenase [10] and α-hydroxyglutarate oxido-reductase [11] resemble nicotinic acid hydroxylase. Both have been shown to be linked to an electron transport chain comprising cytochromes *b* and *c* only [11, 12]. Pipecolate dehydrogenase, partially purified by extraction with sodium taurocholate, was inactivated by precipitation in acid-ammonium sulphate, but, like nicotinic acid hydroxylase, could not be reactivated by incubation with flavin nucleotides [10]. The preparation contained

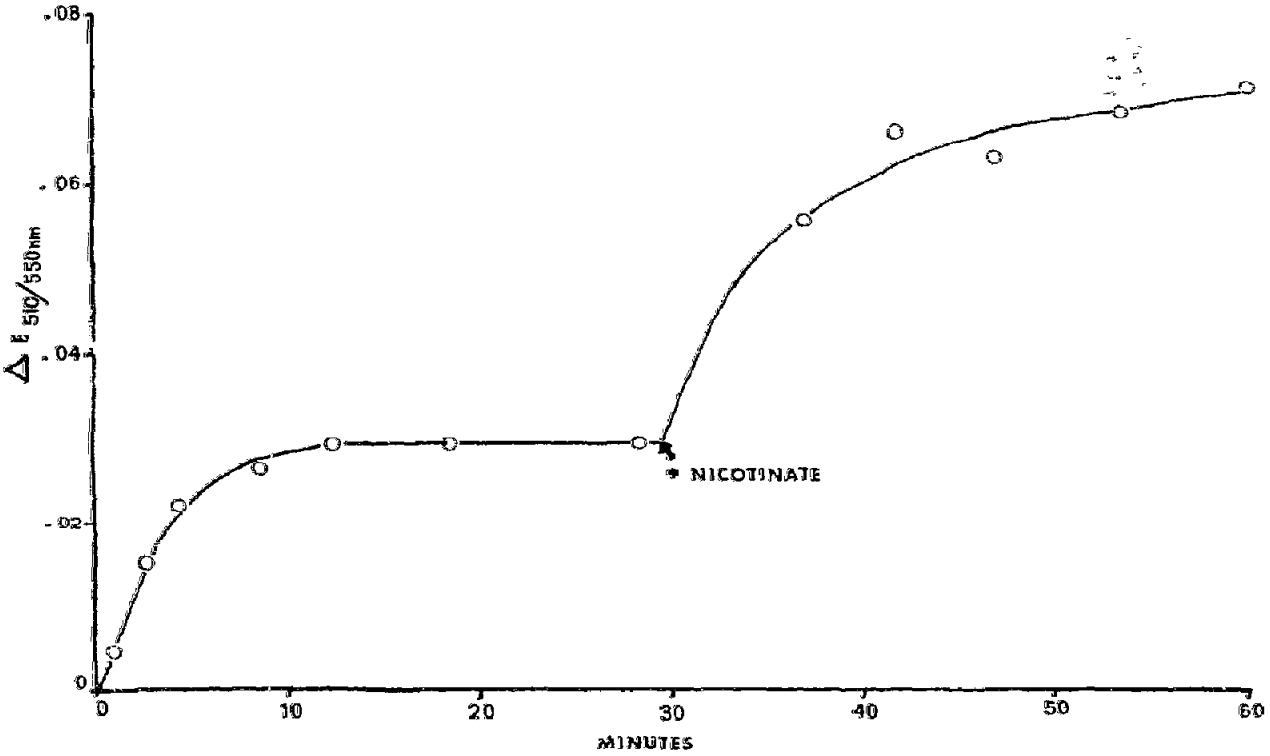

Fig. 3. Reaction of *o*-phenanthroline with the solubilised nicotinic acid hydroxylase. 50 µl of 50 mM *o*-phenanthroline in 20% aqueous ethanol were added to 1 ml enzyme preparation (2.5 mg/ml protein). Spectra were recorded between 400 and 600 nm, against a blank without *o*-phenanthroline, over a 30 min period and the difference in absorbance between 510 nm and 550 nm plotted against time. After 30 min 20 µmoles Na-nicotinate were added to both cuvettes and the change in absorbance followed for a further 30 min.

cytochrome *b* but no evidence was presented for its reduction by pipecolate. Similarly no direct evidence that α-hydroxy glutarate oxidoreductase was a flavoprotein, could be obtained [11]. Substrate reduced spectra of cell wall/membrane [3, 11] or electron transport particles [12] are similar for all three substrates, suggesting a common linkage of these inducible enzymes to the cytochromes, which is different from that of the constitutive respiratory enzymes such as succinate, or NADH dehydrogenases.

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