THE EFFECTS OF TRYPSIN ON THE MEMBRANE-BOUND NICOTINIC ACID OXIDASE IN *PSEUDOMONAS OVALIS* CHESTER

M.V. JONES

*Department of Microbiology, University College, Newport Road, Cardiff, CF2 1TA, Wales, U.K.**

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1. Introduction

The organisation of functional enzyme complexes is probably very important to the general structure of the membrane [1]. An examination of specific complexes may therefore contribute considerably to our understanding of membrane architecture. Nicotinic acid oxidase is a membrane-bound enzyme complex in *Pseudomonas ovalis* Chester [2]. The effect on this system of a number of membrane disruptive agents such as detergents, proteases, and phospholipases is being studied. The properties of a detergent-solubilzied nicotinic acid hydroxylase–cytochrome *c* complex have been described [3]. The effects of trypsin on the membrane nicotinic acid oxidase are now reported.

2. Materials and methods

Growth of *Ps. ovalis* Chester on nicotinic acid and the preparation of the cell wall–membrane fraction have been described [2]. Membrane fractions were resuspended in 50 mM Tris–HCl buffer, pH 7.6, for trypsin digestion.

To prepare trypsin-solubilised nicotinic acid hydroxylase, 10 ml cell wall–membrane fraction was treated with 100 μg/ml trypsin for 1 hr at 30°C, and then centrifuged at 100 000 *g* for 1 hr at 4°C. The supernatant was carefully decanted and solid $(NH_4)_2SO_4$ added slowly, with constant stirring, until 80% saturated. After standing in ice for 30 min the small amount of brownish precipitate was collected by centrifugation and redissolved in 2 ml 50 mM Tris–HCl buffer pH 7.4.

Nicotinic acid oxidase was measured using an oxygen electrode [2]. Nicotinic acid hydroxylase was assayed spectrophotometrically using either DICPIP [3] or potassium ferricyanide as acceptors. The latter assay contained, in 3 ml total volume: 150 μmoles potassium phosphate buffer, pH 7.2; 30 μmoles potassium ferricyanide; 30 μmoles sodium nicotinate, and 50–100 μl membrane preparation. Decrease in absorbance at 450 nm was followed using cuvettes of 1 cm light path. Nicotinate was omitted from the reference cuvette. Protein was measured by the method of Lowry et al. [4].

Cytochrome *c* (horse heart type III), trypsin (bovine pancreas type III), and trypsin inhibitor (lima bean type II-L) were obtained from Sigma (London) Chemical Co. U.K., and all other chemicals from B.D.H. Chemicals, Poole, Dorset, U.K.

3. Results

3.1. *Effect of trypsin on membrane nicotinic acid oxidase*

The cell wall–membrane fraction was incubated for 5 min at 30°C with varying concentrations of trypsin. An equivalent of trypsin inhibitor was added and the mixture centrifuged at 100 000 *g* for 1 hr to obtain residual membrane and supernatant fractions. Nicotinic acid oxidase and hydroxylase was assayed in each fraction.

The results (fig. 1) indicate that loss of oxidase activity with increasing concentrations of trypsin is accompanied by an equivalent loss of membrane

* Address for correspondence: School of Biological Sciences, University of East Anglia, Norwich, NOR 88C, U.K.

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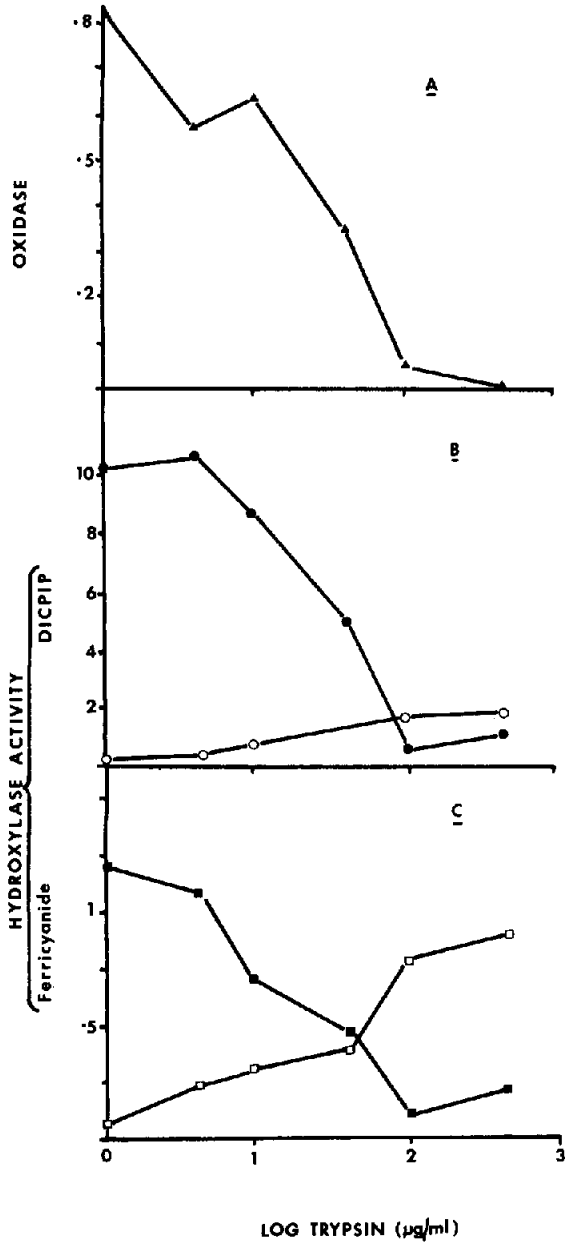

Fig. 1. Effect of trypsin on membrane nicotinic acid oxidase and hydroxylase. CEll wall–membrane (6 mg/ml protein) was incubated with up to 500 μg/ml trypsin at 30°C for 5 min. A) (▲—▲—▲) membrane nicotinic acid oxidase (μmoles O_2 uptake/min/ml). B) DICPIP coupled nicotinic acid hydroxylase (ΔE_{600}/min/ml) in membrane (●—●—●) and supernatant (○—○—○). C) Ferricyanide coupled nicotinic acid hydroxylase (ΔE_{450}/min/ml) in membrane (■—■—■) and supernatant (□—□—□).

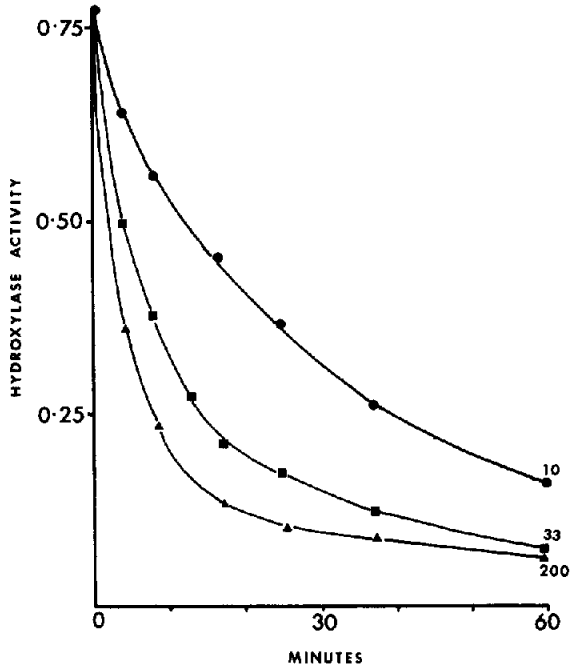

Fig. 2. Effect of varying buffer molarity on the rate of trypsin digestion. Membrane fraction was resuspended in Tris–HCl buffer, pH 7.6, at three different ionic strengths, 10 mM (●—●—●), 33 mM (■—■—■), 200 mM (▲—▲—▲), and incubated with 25 μg/ml trypsin. Samples (50 μl) were withdrawn at time intervals for measurement of DICPIP-coupled nicotinic acid hydroxylase (ΔE_{600}/min).

hydroxylase. When the hydroxylase is coupled to DICPIP, very little activity is detected in the supernatant (fig. 1b) but when ferricyanide is used as the acceptor considerable (73%) hydroxylase activity appears in the supernatant (fig. 1c). Whereas only 16% of the total ferricyanide coupled hydroxylase activity is lost on trypsin digestion, 78% of the DICPIP coupled activity and 100% of the oxidase activity is not recoverable after 5 min incubation with 500 μg/ml trypsin.

In mitochondria, substrates can protect against the action of proteases [5] and in yeast cytochrome b_2 both presence of cytochrome *c* (a substrate) and different molarity buffers affected the rate of trypsin degradation [6]. In the present system the rate of loss of DICPIP coupled hydroxylase activity in the presence of trypsin was greater in 200 mM than in 10 mM Tris buffer (fig. 2) but the addition of nicotinate had no effect.

Table 1
Solubilisation of nicotinic acid hydroxylase by trypsin.

	Hydroxylase activity	
	Ferricyanide	DICPIP
Untreated membrane	2.21	0.367
Trypsin-treated membrane	0.37	0.012
Soluble trypsin extract	6.10	0.055

Membrane (5 mg/ml protein) was incubated with 100 μg/ml trypsin for 1 hr and the trypsin extract concentrated as described in Materials and methods. Enzyme activity is given as μmoles acceptor reduced/min/mg protein.

It has also been observed that the nicotinic acid oxidase activity of membrane 'ghosts', prepared by the osmotic lysis of spheroplasts [7], was resistant to trypsin treatment. This is probably because the hydroxylase is exposed on the inner surface of the vesicle membrane which would be impermeable to trypsin. NADH oxidase is similarly insensitive to phospholipase C in 'ghost' preparations while being rapidly inactivated in cell wall–membrane fractions [8].

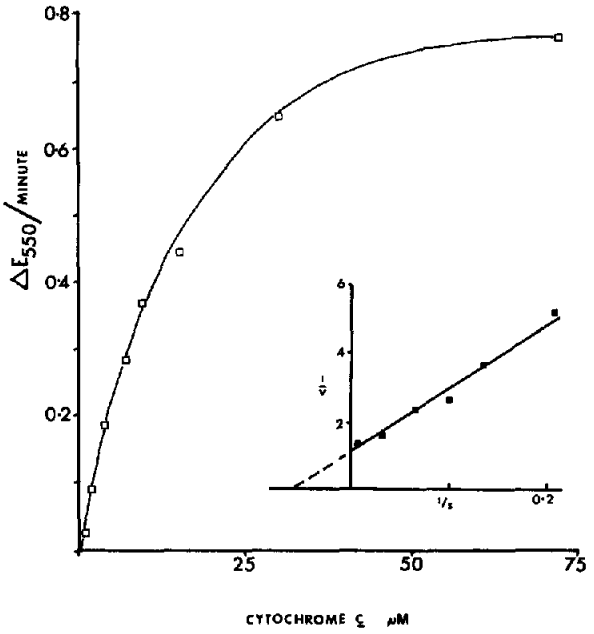

Fig. 3. Reaction of trypsin solubilised nicotinic acid hydroxylase with cytochrome *c*. Reaction mixture contained in 3 ml, 50 μl trypsin solubilised hydroxylase, 30 μmoles Tris–HCl buffer pH 7.4, 30 μmoles sodium nicotinic, and varying concentrations of mammalian cytochrome *c*. Change in absorbance at 550 nm was followed. Reference cuvette lacked nicotinate. Inset shows soluble reciprocal plot.

3.2. *Properties of the trypsin solubilised hydroxylase*

During the preparation of the trypsin-solubilised nicotinic acid hydroxylase it was noted that the ammonium sulphate precipitate redissolved very easily in Tris buffer in contrast to the detergent-solubilised complex previously described [3]. This present preparation had 3 times the ferricyanide coupled hydroxylase activity of the original membrane (table 1). Between 30–40% of the membrane protein appeared to be released by trypsin but both NADH and succinate dehydrogenase remained in the residual membrane along with the bulk of the cytochromes.

The solubilised hydroxylase contained traces of cytochrome *c* but this was not reducible by nicotinate again in contrast to the detergent-solubilised complex. The trypsin-solubilised hydroxylase readily reduced exogenous cytochrome *c* (fig. 3). A K_M of 2×10^{-5} M for cytochrome *c* was determined.

4. Discussion

Trypsin can affect specific components of the mitochondrial electron transport chain [5] and has been used to investigate the structure of chloroplast thylakoids [9]. This paper reports some effects of trypsin on a bacterial membrane enzyme complex. The solubilisation of nicotinic acid hydroxylase by trypsin was unexpected. In previous work the enzyme was found to be very difficult to solubilise without using detergent and lipase [3]. This suggested that hydrophobic bonds were important in the enzyme–membrane association and that the hydroxylase was a typical 'intrinsic' membrane protein [1]. Trypsin, however, is a large water-soluble enzyme that has been shown to act on proteins on the membrane surface [9]. These observations are best explained if the hydroxylase is considered to be an amphipathic similar to microsomal cytochrome b_5 and cytochrome b_5 reductase which could be split into a hydrophobic active segment and hydrophobic tail by hydrolytic enzymes [10, 11].

In *Ps. ovalis* membranes the site of action of trypsin appears to be between the nicotinic acid hydroxylase

and cytochrome *c*. It is not known, at present, whether trypsin cleaves part of the hydroxylase molecule, a specific binding protein, or a component of the oxidase e.g. a non-haeme iron protein. Although up to 40% of the membrane protein can be solubilised by trypsin, nicotinic acid hydroxylase is the only active enzyme so far identified. The nature of the other protein is to be investigated.

Acknowledgement

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