Pseudomonas aeruginosa cholinesterase and phosphorylcholine phosphatase: two enzymes contributing to corneal infection

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1. SUMMARY

Choline, acetylcholine and betaine used as the sole carbon, nitrogen or carbon and nitrogen source increase cholinesterase activity in addition to phosphorylcholine phosphatase and phospholipase C activities in Pseudomonas aeruginosa. The cholinesterase activity catalyses the hydrolysis of acetylthiocholine (K_m approx. 0.13 mM) and propionylthiocholine (K_m approx. 0.26 mM), but not butyrylthiocholine, which is a pure competitive inhibitor (K_i 0.05 mM). Increasing choline concentrations in the assay mixture decreased the affinity of cholinesterase for acetylthiocholine, but in all cases prevented inhibition raised by high substrate concentrations. Considering the properties of these enzymes, and the fact that in the corneal epithelium there exists a high acetylcholine concentration and that Pseudomonas aeruginosa produces corneal infection, it is proposed that these enzymes acting coordinately might contribute to the breakdown of the corneal epithelial membrane.

2. INTRODUCTION

Pseudomonas aeruginosa is an opportunistic bacterium which elaborates several extracellular factors that contribute to its pathogenesis [1]. One of these virulence factors has been identified as phospholipase C [2]. Several authors have shown that this enzyme and alkaline phosphatase are produced only in a medium containing a low phosphate concentration [2–5]. However, we have recently shown that P. aeruginosa produces phospholipase C, but not alkaline phosphatase, when grown on choline or betaine as the sole carbon and nitrogen source, and in the presence of a high phosphate concentration [6]. Since under the same culture conditions P. aeruginosa synthesizes...
an acid phosphatase which was identified as a phosphorylcholine phosphatase [6-8], it was postulated that the coordinated action of \textit{P. aeruginosa} phospholipase C and acid phosphatase might contribute to the breakdown of the host cell's membrane phospholipids. Considering that a cholinesterase activity is also increased [7,9,10], we focussed our attention on its possible role in collaboration with phospholipase C and phosphorylcholine phosphatase in the production of the corneal infection.

Certain properties of these enzymes, particularly of cholinesterase described below, may explain how the high acetylcholine concentration present in the corneal epithelium [11] and literature cited therein), may contribute to the \textit{P. aeruginosa} corneal infection.

3. MATERIALS AND METHODS

3.1. Organism and growth conditions

\textit{Pseudomonas aeruginosa} (NCTC, Fieldes III, 1924, U.K.) was grown aerobically at 37 °C in a basal salt medium as previously described [6]. 20 mM acetylcholine, choline or betaine were utilized as the sole carbon, nitrogen or carbon and nitrogen source. When necessary 20 mM NH₄Cl or succinate were utilized as the preferential nitrogen or carbon sources, respectively.

3.2. Enzyme preparation and assays

Crude periplasmic extracts [9] or partially purified enzymes from these extracts [12] were utilized as the source of cholinesterase and acid phosphatase. Kinetic constants obtained with any of the above cholinesterase preparations were identical to those obtained with the purified enzyme as described earlier [13].

Cholinesterase activity was assayed with acetylthiocholine or propionylthiocholine as substrates as described [9,14]. The pH, 6.0 or 8.0, of the reaction mixture was maintained with 70 mM phosphate buffer. The acid phosphatase activity was assayed with the sodium salt of \textit{p}-nitrophenyl phosphate [9] or phosphorylcholine as substrates [7]. The product formation was detected by measuring \textit{p}-nitrophenol or inorganic phosphate (Pᵢ).

The phosphate released was measured chemically as described [15].

The kinetic constants were calculated as described earlier [16,17].

The action of \textit{P. aeruginosa} phospholipase C and acid phosphatase on \textit{[³²P]phosphatidylcholine} was performed as described in reference [6], except that in this case both enzymes and 2 mM MgCl₂ were added at the beginning of the incubation period and the reaction was carried out at pH 7.4 without previous adjustment to pH 5.0.

4. RESULTS AND DISCUSSION

A noticeable increase of cholinesterase and acid phosphatase activities can be detected when \textit{P. aeruginosa} utilizes choline, betaine or acetylcholine as the sole carbon, nitrogen or carbon and nitrogen source. In all cases the specific activity of cholinesterase and acid phosphatase found in periplasmic extracts (obtained from bacteria at the end of the exponential phase, subsequently treated with EDTA-lysozyme [9]) was in the range of 200-300 nmol min⁻¹ (mg protein)⁻¹. Experiments similar to those described by Lisa et al. [9] indicated that a restriction of the increment of cholinesterase and acid phosphatase activities may occur only if the preferential carbon (succinate) and nitrogen (NH₄Cl) sources are simultaneously present.

The optimal pH for \textit{P. aeruginosa} cholinesterase was found to be between 6.0 and 8.0. Table 1 shows that, at both pH values, cholinesterase catalyses the hydrolysis of acetylthiocholine and propionylthiocholine but not butyrylthiocholine. Data obtained from saturation curves of this enzyme by acetylthiocholine in the presence or different concentrations of butyrylthiocholine revealed that this compound was a pure competitive inhibitor of cholinesterase. The \textit{Kᵢ} value for butyrylthiocholine, calculated from replots of the primary reciprocal plots was about 0.05 mM. Although the \textit{Vₘₐₓ} with propionylthiocholine was about 50-60% lower than that obtained with acetylthiocholine, considering the \textit{Kₘ} values, the catalytic efficiency (\textit{Vₘₐₓ}/\textit{Kₘ}) of the enzyme against both substrates and the fact that


butyrylthiocholine was not a substrate, the *P. aeruginosa* enzyme may be considered an acetylcholinesterase.

Fig. 1 shows the saturation curves of *P. aeruginosa* acetylcholinesterase by acetylthiocholine in the presence of different choline concentrations (indicated on each curve). Inset shows double reciprocal plots of the same data. Enzyme activity was measured at 37 °C in the presence of 70 mM sodium phosphate buffer, pH 6.0, and 0.33 mM 5,5'-dithiobis-(2-nitrobenzoic acid). The change in absorbance was followed at 412 nm during the first 2 min of the reaction. Similar results were obtained at pH 8.0. Data correspond to a representative experiment.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Acetylthiocholine</th>
<th>Propionylthiocholine</th>
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</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.0</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>8.0</td>
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<tr>
<td>( K_m ) (mM)</td>
<td>0.100</td>
<td>0.135</td>
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<tr>
<td></td>
<td>0.133</td>
<td>0.133</td>
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<tr>
<td>( V_{max} ) (nmol mg protein(^{-1}) min(^{-1}))</td>
<td>1353</td>
<td>2660</td>
</tr>
<tr>
<td></td>
<td>2707</td>
<td>2707</td>
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<tr>
<td>( V_{max}/K_m )</td>
<td>13530</td>
<td>19703</td>
</tr>
<tr>
<td></td>
<td>20353</td>
<td>20353</td>
</tr>
<tr>
<td>( K_{1/2} ) (mM)</td>
<td>5.2</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>1.4</td>
<td>nd</td>
</tr>
</tbody>
</table>

\( K_{1/2} \) and \( V_{max} \) values were calculated from double reciprocal plots of saturation curves by different substrates. Substrate concentrations utilized were in the range of 0.05 mM to 0.5 mM. The \( V_{max} \) is expressed as nmol of hydrolysed substrate min\(^{-1}\) (mg protein\(^{-1}\)). \( K_{1/2} \) is the constant of the Hill equation (\( \log v / V_{max} - v = - n \log S + \log K' \)) and is defined as the acetylthiocholine concentration that produces 50% inhibition of the reaction rate. Their values were calculated by utilizing substrate concentrations (\( S \)) in the range of 0.5 to 8 mM. Plots of \( 1/v \) vs. these acetylthiocholine concentrations resulted in parabolic curves. The constants represent an average from at least three independent experiments performed with partially purified enzyme obtained from cells of different cultures.

\( ^{a} \) nd = not determined.
higher activity was also observed at about pH 5.0, the enzyme exerted its action with more than 80% efficiency in the pH range of 5.0–8.5. The acid phosphatase action against its natural substrate in a broad pH range was indicative that this enzyme could work in parallel with the phospholipase C to break down phospholipids containing choline. This was confirmed by an experiment similar to that described by Lucchesi et al. [6], but in this case performed at pH 7.4. The formation of \( R_1 \) and choline was detected after the simultaneous action of partially purified \( P. aeruginosa \) phospholipase C and acid phosphatase against phosphatidylcholine. Identical results were obtained with \( P. aeruginosa \) culture supernatants (as source of PLC) and periplasmic extracts (as source of acid phosphatase) from bacteria grown on choline as the sole carbon and nitrogen source.

Considering its periplasmic localization [7,18] and the fact that the \( P. aeruginosa \) cholinesterase may be an acetylcholinesterase, it is hardly likely that \( P. aeruginosa \) synthesizes this enzyme to control its intracellular acetylcholine level. It is more likely that acetylcholinesterase is synthesized by the bacterium to cover its need for carbon, nitrogen, or carbon and nitrogen through the acetylcholine hydrolysis of its environment. Since the corneal epithelium has a high acetylcholine concentration and the \( P. aeruginosa \) produces corneal infection [1], we feel that our data and those previously cited [6,7,9,10] may be extrapolated to suggest an explanation for the role that choline derivatives, the enzymes cholinesterase, acid phosphatase and phospholipase C may play in such an infection.

After corneal injury or any other cause which permits the adherence of \( P. aeruginosa \) to the wounded cornea, the bacterium encounters a high acetylcholine concentration (up to 200 \( \mu \)g/gm in the corneal epithelium; [11] and literature cited therein) which may be utilized for its growth and a parallel increase of the cholinesterase activity.

At physiological pH values this enzyme catalyses the hydrolysis of acetylcholine to produce choline. This compound can: (a) positively modify the kinetics of the acetylcholinesterase, fundamentally if high substrate concentrations are present; (b) be efficiently transported into the bacteria through the uptake systems [19]; and (c) support bacterial growth and increase, in addition to cholinesterase, the phospholipase C and phosphorylcholine phosphatase activities [6,7]. These two enzymes acting coordinately, and under conditions apparently not adversely affecting the action of either enzyme (in a similar pH-range and even in the presence of \( P_1 \)), might contribute to the breakdown of the corneal epithelial membrane.

As stated in [1]: “certainly, a number of questions remain regarding the pathogenesis of \( P. aeruginosa \) disease”, however, we feel that through studying the choline metabolism it is possible to obtain other perspectives to understand the establishment and maintenance of the infection caused by this organism.

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