Physiological regulation, purification and properties of urease from Methylophilus methylotrophus

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Abstract

The methylotrophic bacterium Methylophilus methylotrophus hydrolyses urea to ammonia using a cytoplasmic urease (EC 3.5.1.5). During growth in continuous culture under various nutrient limitations, urease was induced by urea and short-chain amides (formamide and urea -> acetamide), and repressed by excess ammonia. The enzyme was purified using ammonium sulfate fractionation and fast protein liquid chromatography (FPLC), and exhibited a narrow substrate specificity (urea -> formamide and acetamide; no activity with other amides) with a \( K_m \) for urea of 3.8 mM. Gel filtration FPLC and SDS-PAGE showed that the enzyme had a native \( M_r \) of approximately 190 000 and was composed of \( \alpha \) (\( M_r \) 64 000), \( \beta \) (\( M_r \) 15 500) and \( \gamma \) (\( M_r \) 15 000) subunits in the probable ratio \( \alpha_2 \beta_2 \gamma_2 \). The physiological regulation and biochemical properties of the M. methylotrophus urease are compared with those of other bacterial ureases. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

Keywords: Methylophilus methylotrophus; Urease; Physiological regulation; Purification and properties

1. Introduction

The methylotrophic bacterium Methylophilus methylotrophus uses a relatively wide range of nitrogen sources for growth including ammonia, short-chain amides and urea. Formamide and acetamide are hydrolysed to ammonia and the corresponding organic acid using formamidase (formamide amido-

Abbreviations: D, dilution rate; DTT, dithiothreitol; FPLC, fast protein liquid chromatography

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regulation of urease expression has been investigated (albeit mainly using imprecisely defined batch cultures) (see [5,6]).

There is some physiological evidence for high-affinity, energy-dependent urea uptake by *Alcaligenes eutrophus*, *Klebsiella pneumoniae* and *Bacillus megaterium* [7,8], and also by *M. methylotrophus* [9]. In addition, genes encoding components of putative active transport systems for short-chain amides or urea in *Helicobacter pylori*, *Mycobacterium smegmatis*, *Pseudomonas aeruginosa* and *Rhodococcus* sp. R312 have been cloned and sequenced (see [10–13]), and both an outer-membrane porin and a binding protein-dependent, active transport system for short-chain amides and urea have been characterised from *M. methylotrophus* [9,14]. The latter two transport systems support the reported presence of a urease in *M. methylotrophus* (see [2]).

This paper describes the physiological regulation of urease expression in *M. methylotrophus* during growth in continuous culture, together with the purification and properties of the enzyme.

2. Materials and methods

2.1. Growth of *M. methylotrophus*

*M. methylotrophus* (NCIMB 10515) was grown in continuous culture (37°C, pH 6.8) in methanol-ammonia-salts medium under various nutrient limitations with methanol as the carbon source and ammonia, formamide, acetamide or urea as the nitrogen source [1,3,14]. Culture supernatants were prepared and assayed for ammonia as described previously [1,3].

2.2. Preparation of washed cells and a high-speed supernatant fraction

Washed cells and a high-speed supernatant fraction were prepared as described previously [14].

2.3. Purification of urease

Urease was purified from a high-speed supernatant fraction prepared from a urea-limited continuous culture (D 0.1 h⁻¹) essentially as described previously for formamidase [3,4] except that the 20 mM Bis-Tris buffer pH 6.8 was supplemented with 1 mM DTT, and a 35–65% ammonium sulfate fraction was collected and dialysed prior to FPLC.

2.4. Other methods

Urease activity with urea and various short-chain amides was measured colorimetrically at 37°C, pH 7.0 as described previously [9]; the activity with urea was expressed as the half of the measured rate of ammonia production. The $K_m$ for urea was measured using urea concentrations in the range 0.1–5.0 mM and calculated from a plot of $v$ against $s$ using the KFitSim programme. SDS-PAGE was carried out as described previously [1]. Gels were stained for protein with Kenacid blue R and, where appropriate, subjected to image analysis using a GDS2000 gel documentation system (Ultraviolet Products) linked to a PC. For N-terminal amino acid sequencing, an SDS-polyacrylamide gel containing purified urease was blotted on to a poly(vinylidine difluoride) membrane, then the $\alpha$-subunit was excised and sequenced as described previously [4].

2.5. Presentation of results

Results are expressed, where appropriate, as the mean ± S.E. with the number of independent determinations shown in brackets.

3. Results

3.1. Physiological regulation of urease expression in *M. methylotrophus*

*M. methylotrophus* was grown in continuous culture (D 0.10 h⁻¹) under various nutrient limitations with methanol as the carbon source and either ammonia, formamide, acetamide or urea as the nitrogen source. Growth under nitrogen limitation was characterised by low residual concentrations of ammonia, whereas growth under methanol limitation was characterised by high residual concentrations of ammonia (resulting either from the direct addition of excess ammonia or from the hydrolysis of excess amides or urea, depending on the nature of the nitro-
gen source). Urease activities of washed cells were several-fold higher following growth under formamide or urea limitation than under acetamide, ammonia or methanol limitation (Table 1). Overall, therefore, these results indicated that urease was strongly induced by urea and formamide (but not by general nitrogen starvation, i.e. by ammonia limitation), and repressed by high concentrations ammonia. The high urease activity exhibited by formamide-limited or urea-limited cells thus reflected the presence of sufficient formamide or urea to bring about maximum induction, and insufficient ammonia to cause significant repression. Urease activity was not increased by the addition of Ni\(^{2+}\) to the growth medium.

### 3.2. Purification and properties of urease

Urease was purified from a formamide-limited continuous culture of *M. methylotrophus* (D 0.10 h\(^{-1}\)) using cell fractionation, ammonium sulfate fractionation and FPLC. Washed cells resuspended in 20 mM Bis-Tris buffer pH 6.8 lost up to 50% urease activity on freezing and thawing, storage at 4°C or breakage. This lability to freezing and thawing or storage, but not to cell breakage, was decreased by supplementing the buffer with 1 mM DTT. However, the latter failed to prevent further activity being lost during the purification procedure, and the purified enzyme exhibited a specific activity (6.6 \(\mu\)mol min\(^{-1}\) [mg protein]\(^{-1}\)) which was quite low compared with many other bacterial ureases. The enzyme was shown by native and SDS-PAGE to be \(>95\%\) pure.

Purified urease exhibited simple Michaelis-Menten kinetics (\(K_m\) for urea 3.8 mM) and a very narrow substrate specificity (urea \(\approx\) formamide and acetamide; no activity with acrylamide, propionamide or butyramide) (Table 2).

SDS-PAGE of the purified enzyme showed the presence of three subunits of \(M_r\) 64 000 (\(\alpha\)), 15 500 (\(\beta\)) and 15 000 (\(\gamma\)) (Fig. 1). Image analysis showed

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Limiting nutrient</th>
<th>[Ammonia] (mM)</th>
<th>Urease activity ((\mu)mol min(^{-1}) [mg cells](^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>Ammonia</td>
<td>(&lt;0.01)</td>
<td>0.15 (\pm) 0.04 (6)</td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
<td>5.90</td>
<td>0.06 (\pm) 0.03 (3)</td>
</tr>
<tr>
<td>Formamide</td>
<td>Formamide</td>
<td>(&lt;0.03)</td>
<td>1.09 (\pm) 0.13 (7)</td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
<td>8.90</td>
<td>0.21 (\pm) 0.01 (2)</td>
</tr>
<tr>
<td>Acetamide</td>
<td>Acetamide</td>
<td>(&lt;0.03)</td>
<td>0.26 (\pm) 0.06 (4)</td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
<td>7.00</td>
<td>0.07 (\pm) 0.00 (2)</td>
</tr>
<tr>
<td>Urea</td>
<td>Urea</td>
<td>(&lt;0.03)</td>
<td>1.05 (\pm) 0.01 (2)</td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
<td>1.80</td>
<td>0.14 (\pm) 0.01 (2)</td>
</tr>
<tr>
<td>(+Ni\Cl_2 (100 \muM))</td>
<td>Urea</td>
<td>(&lt;0.03)</td>
<td>0.91 (\pm) 0.02 (2)</td>
</tr>
</tbody>
</table>

*M. methylotrophus* was grown in continuous culture (D 0.10 h\(^{-1}\)) under various nutrient limitations with methanol as the source of carbon and ammonia, acetamide, formamide or urea as the source of nitrogen. Washed cells were prepared and assayed for urease activity, and culture supernatants for ammonia concentration, as described in Section 2. Activities are expressed as the mean \(\pm\) S.E. (number of independent determinations).
that the subunits comprised 66%, 17% and 17% respectively of the total protein, indicating a 1:1:1 molar ratio. Gel filtration FPLC yielded a native \( M_r \) of approximately 190,000, suggesting a probable subunit composition of \( \alpha_2\beta_2\gamma_2 \) (\( M_r \) 189,000).

The N-terminal amino acid sequence of the \( \alpha \)-subunit of the purified enzyme (11 residues) lacked the expected N-terminal methionine residue found in other ureases, presumably due to intracellular processing or loss during the purification procedure. However, apart from this, the sequence was similar to that of several other bacterial ureases, particularly those from \( B. \) pasteurii (30% identical), \( S. \) xylosus (27% identical) and \( R. \) melliloti (27% identical) (Fig. 2).

4. Discussion

Previous physiological studies of urease expression in bacteria, mainly using imprecisely defined batch cultures, have indicated that bacterial ureases are either constitutive, or are induced by urea and/or general nitrogen limitation and repressed by ammonia (see [6]). The continuous culture studies described in this paper clearly show that \( M. \) methylotrophus urease is induced by potential substrates such as urea and short-chain amides (formamide and acetamide limitation) rather than by general nitrogen limitation (ammonia limitation), and is repressed by excess amounts of its reaction product, ammonia. Physiological regulation of urease expression in \( M. \) methylotrophus is therefore very similar to that of formamidase, the amide-urea porin and the amide-urea binding protein [3,4,9,14].

Although \( M. \) methylotrophus urease is substantially more labile than other bacterial ureases, its substrate specificity (urea \( \rightarrow \) formamide and acetamide; no activity with other amides) is characteristically narrow (see [7,8]). The high \( K_m \) for urea (3.8 mM) is within the range exhibited by other bacterial ureases and, given that the residual concentration of urea in a urea-limited continuous culture (\( D \) 0.1 h\(^{-1} \)) of \( M. \) methylotrophus is \( < 50 \mu M \), explains the presence of the recently characterised active transport system for urea and short-chain amides in this organism [9].

\( M. \) methylotrophus urease is a three-subunit enzyme of the type found in most urease-positive bacteria (e.g. \( K. \) aerogenes, \( P. \) spp., \( U. \) urealyticum, \( R. \) melliloti, \( S. \) xylosus and \( B. \) pasteurii), rather than a two-subunit enzyme of the type found in \( H. \) spp. It differs slightly from other three-subunit ureases in terms of its native \( M_r \) (190,000 cf. 200,000–250,000) and subunit \( M_r \) (\( \alpha \) 64,000 cf. 60,000–72,000; \( \beta \) 15,500 cf. 10,000–15,000; \( \gamma \) 15,000 cf. 8000–11,000) values, but has an identical subunit composition to that of the recently crystallised enzyme from \( K. \) aerogenes (\( \alpha_2\beta_2\gamma_2 \)) [5,15].

The primary sequence of the \( M. \) methylotrophus urease (measured as the N-terminal amino acid sequence of the \( \alpha \)-subunit of ureases from \( M. \) methylotrophus and six other species of bacteria. The sequence of the \( M. \) methylotrophus enzyme was determined by direct analysis of the purified enzyme as described in Section 2; the other sequences are deduced sequences taken from [5]. Residues identical to those present in the \( M. \) methylotrophus enzyme are shown in bold.

![Fig. 2. N-terminal amino acid sequence alignment of the \( \alpha \)-subunit of ureases from \( M. \) methylotrophus and six other species of bacteria. The sequence of the \( M. \) methylotrophus urease was determined by direct analysis of the purified enzyme as described in Section 2; the other sequences are deduced sequences taken from [5]. Residues identical to those present in the \( M. \) methylotrophus enzyme are shown in bold.](https://academic.oup.com/femsle/article-abstract/160/1/131/514384)

Table 2

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (%)</th>
</tr>
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<tbody>
<tr>
<td>Urea</td>
<td>100</td>
</tr>
<tr>
<td>Formamide</td>
<td>14</td>
</tr>
<tr>
<td>Acetamide</td>
<td>11</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>0</td>
</tr>
<tr>
<td>Propionamide</td>
<td>0</td>
</tr>
<tr>
<td>Butyramide</td>
<td>0</td>
</tr>
</tbody>
</table>

Urease was purified from \( M. \) methylotrophus and assayed with various substrates (50 mM) as described in Section 2.
Acknowledgments

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References


