Urease Activity of the Cyanobacterium *Anabaena cylindrica*

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The cyanobacterium *Anabaena cylindrica* was found to have nickel-dependent urease activity. Addition of nickel to a nickel-free culture led to recovery of urease activity. This was not prevented by chloramphenicol. Cell-free extracts of nickel-depleted cells did not regain activity upon addition of Ni^{2+} . Enzyme synthesis was constitutive; similar urease activities occurred in urea-, NO_3^- or NH_3 -grown cultures or those grown under N_2 -fixing conditions. The urease activity had hyperbolic kinetics, with an apparent K_m with respect to urea of 1.3 ± 0.6 (SD) mM. Enzyme activity was enhanced up to two fold by MgSO_4 and MnSO_4 at pH 7.5. At 10 mM-urea, cells hydrolysed urea and excreted NH_3 in excess of biosynthetic requirements.

INTRODUCTION

Cyanobacterial species have been reported to grow on urea as a nitrogen source (Fogg *et al.*, 1973) but were first shown to possess urease (EC 3.5.1.5) activity by Berns *et al.* (1966). An alternative urea-degrading enzyme, urea amidolyase (EC 6.3.4.6), has been observed in yeasts and some unicellular green algae (Roon & Levenberg, 1968, 1972); its presence explains the ability of these organisms to hydrolyse urea without a detectable urease.

The two enzymes differ markedly in their mechanisms and co-factor requirements. Urease requires no co-factors for urea hydrolysis while urea amidolyase is biotin-dependent and requires ATP, Mg^{2+} and CO_2 or bicarbonate (Roon & Levenberg, 1970). Urea amidolyase has two enzyme activities: urea carboxylase, which forms allophanate in an ATP-dependent carboxylation reaction, and allophanate hydrolase (Syrett & Leftley, 1976). On the basis of these differences Syrett & Leftley (1976) proposed three criteria for distinguishing urease and urea amidolyase in algal extracts: inhibition of urease by hydroxyurea, inhibition of urea amidolyase by avidin and the reversal of this inhibition by addition of excess biotin. In this paper we report the application of these criteria to urea hydrolysis by extracts of the cyanobacterium *Anabaena cylindrica*. Dixon *et al.* (1975) found that urease from jack beans is a nickel-containing metalloenzyme and subsequently other ureases have also been shown to be nickel-dependent (Schneider & Kaltwasser, 1984). We have investigated the nickel-dependence of urea hydrolysis in *A. cylindrica*.

METHODS

Cyanobacterial growth. *A. cylindrica*-ATCC 27899 was obtained from the American Type Culture Collection and grown as described by Daday *et al.* (1977) in the modified medium of Allen & Arnon (1955). Cells were grown either under N_2 -fixing conditions in the absence of a combined nitrogen source or in the presence of urea (2 mM), $(\text{NH}_4)_2\text{HPO}_4$ (2 mM) or NaNO_3 (4 mM). All components except phosphate and nickel were present at one-eighth the strength used by Allen & Arnon (1955); phosphate was full strength (2 mM) and nickel usually four times full strength (0.68 μM ; variations are specified for individual experiments). For some experiments cells were grown in nickel-depleted medium prepared as described by Daday *et al.* (1985). During growth cells were continuously sparged with 0.3% CO_2 in air at approximately 170 ml min^{-1} under a light intensity of 150 $\mu\text{E s}^{-1} \text{m}^{-2}$ (photosynthetically active radiation). Cells were harvested during exponential growth (less than 200 Klett units; Mallette, 1969).

Assays. Cells were concentrated by centrifugation, resuspended in 50 mM-HEPES/NaOH, 1 mM- Na_2 -EDTA, pH 7.5, and passed through a French pressure cell at 138 MPa. The resulting extract was centrifuged at 13000 *g* for 15 min (4 °C). Supernatants were made 10 mM in urea and 5 mM in MgSO_4 and incubated at 30 °C for 30 min. The reaction was stopped by addition of 1 M- H_2SO_4 (final concentration 10 mM). Released NH_3 was measured with an Orion NH_3 electrode (model no. 95-12, Orion Research) by adding 10 M- NaOH (final concentration 0.1 M) to convert NH_4^+ into free NH_3 . Protein concentrations were measured by the method of Peterson (1977), standardized with bovine serum albumin. Acetohydroxamic acid, hydroxyurea and avidin were obtained from Sigma.

RESULTS AND DISCUSSION

Nature of the urea-degrading enzyme in A. cylindrica

Our results show that *A. cylindrica*, in common with other cyanobacteria tested (Syrett & Leftley, 1976), has urease and not urea amidolyase activity (Table 1). Urea hydrolysis was not affected by avidin but was markedly inhibited by the urease inhibitors acetohydroxamic acid and hydroxyurea. Acetohydroxamic acid is a competitive inhibitor of urease (Kobashi *et al.*, 1962). Hydroxyurea is an inhibitor, but is also a substrate for urease (Reithel, 1971; Blakeley *et al.*, 1969), releasing one molecule of NH_3 per molecule of hydroxyurea. The degree of inhibition thus changes with time of incubation. The activity eluted as a single band from Sephacryl S-300 (Pharmacia) equilibrated with 50 mM-HEPES/NaOH, 1 mM- Na_2 -EDTA, 1 mM-dithiothreitol, pH 7.5 (result not shown), which is also consistent with the presence of a single urea-degrading enzyme.

Nickel-dependence of urease activity

The level of urease activity depended on the concentration of nickel in the medium (Fig. 1). Cells grown in the absence of nickel had negligible activity (typically less than 2% of that observed in cells grown with 0.68 μM - NiSO_4). Addition of NiSO_4 (0.68 μM) to such cultures resulted in the recovery of activity over approximately 24 h (Fig. 2). Chloramphenicol (20 $\mu\text{g ml}^{-1}$) did not prevent the recovery in experiments done over 6 h. After 24 h in the presence of chloramphenicol the cells had an apparently lower specific activity of urease than control cells but this may well have been due to nonspecific toxic effects over the longer time period. When extracts of cells grown in nickel-depleted medium were incubated with 1 μM - or 5 mM- NiSO_4 for 2 h before adding urea no increase in activity was observed. Thus like the jack bean enzyme *A. cylindrica* urease was nickel-dependent (Dixon *et al.*, 1975), but cellular components are required for incorporation of the metal to give an active enzyme.

Properties of the urease activity

The specific activity of urease varied from culture to culture, falling in the range 16 to 58 nmol min^{-1} (mg protein) $^{-1}$. The reason for the variation is not known but it may have reflected subtle variations in growth conditions. The enzyme in crude cell-free extracts had hyperbolic kinetics, indicating the reaction to be pseudo-first-order with respect to urea concentration. An apparent K_m value of 1.3 ± 0.6 (SD) mM was determined. Urease activity in crude extracts was affected by Mg^{2+} and Mn^{2+} in a fashion previously unreported for urease. Increasing concentrations of MgSO_4 in the assay mixture resulted in increasing urease activity. Saturation occurred at approximately 5 mM- MgSO_4 . The effect was more pronounced at pH 7.0 than at pH 8.0. At pH 7.5, 5 mM- MgSO_4 typically produced a 1.6 to 2-fold enhancement of activity. The effect was specific for Mg^{2+} and Mn^{2+} : other metal ions tested (Fe^{3+} , Ni^{2+} , Co^{2+} and Zn^{2+} , each at 10 mM in the assay) were inhibitory (cf. Reithel, 1971).

Regulation of urease synthesis

The recovery of activity when NiSO_4 was added to a nickel-depleted culture was not inhibited by chloramphenicol which suggested that urease protein was synthesized in the absence of Ni^{2+} and that its synthesis was constitutive. The lack of regulation of synthesis was confirmed by the presence of similar levels of urease activity during growth on urea (2 mM), $(\text{NH}_4)_2\text{HPO}_4$ (2 mM), NaNO_3 (4 mM) and under N_2 -fixing conditions (results not shown). Such phenomena have been

Fig. 1

Fig. 2

Fig. 1. Dependence of urease activity in *A. cylindrica* on the Ni^{2+} concentration in the growth medium. Cells were grown in media supplemented with the indicated Ni^{2+} concentration and harvested at 170 Klett units. Extracts were prepared by French pressure cell disruption and urease activity was measured as described in Methods.

Fig. 2. Recovery of urease activity in cells grown in nickel-depleted growth medium. NiSO_4 ($0.68 \mu\text{M}$) was added at zero time and at appropriate times cell extracts were made and assayed (○). A similar experiment was done but with chloramphenicol ($20 \mu\text{g ml}^{-1}$) added immediately before the NiSO_4 (●).

Table 1. *Differentiation between urease- and urea amidolyase-mediated urea hydrolysis in A. cylindrica*

A cell-free extract [specific activity $57 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$] was prepared and the initial rates of enzyme activity were measured in the presence of 10 mM -urea plus the indicated inhibitors. The rate of NH_3 formed from hydroxyurea alone is also shown for a 60 min incubation.

Inhibitor	Concentration	Activity (%)
None	—	100
Acetohydroxamic acid	10 mM	5
Hydroxyurea	10 mM	27
Hydroxyurea without urea	10 mM	4
Avidin	$50 \mu\text{g ml}^{-1}$	100

observed with many cyanobacterial enzymes (Carr, 1973; Doolittle, 1979). Synthesis of a urease apoenzyme in the absence of nickel was observed in soybean seeds; no recovery of urease activity occurred on addition of Ni^{2+} to cell-free extracts (Winkler *et al.*, 1983).

Whole-cell urease activity

When cells were grown in 10 mM -urea significant amounts of NH_3 appeared in the medium (up to 15 mM within 4 d), resulting in cell death (observed as a rapid drop in Klett value, cell lysis and destruction of pigments) in some cases. No NH_3 was released in the absence of urea or when nickel-depleted cells were incubated in urea (results not shown). In cultures grown on 1 mM -urea only transient increases in NH_3 concentration (less than 0.2 mM) were observed. Hence in conditions of high urea concentration the urease rate appeared to be significantly greater than the rate of NH_3 incorporation by glutamine synthetase.

This possibility was tested further in shorter term experiments. With 10 mM -urea a linear rate of NH_3 release into the medium occurred for at least 60 min; the rate was enhanced by only 30% when methionine sulfoximine (1 mM) was added to the cells 3 h before urea addition. Such a preincubation is known to inhibit further incorporation of NH_3 via the glutamine synthetase

reaction (Mackerras & Smith, 1986). In the presence of methionine sulfoximine the rates of NH_3 release from whole cells [$3.2 \text{ nmol min}^{-1} (\text{mg dry wt cells})^{-1}$] were close to (within 20%) those measured in crude cell-free extracts. For such experiments it was important that a control with methionine sulfoximine but no urea also be included since significant amounts of NH_3 are released from cells [typically $0.36 \text{ nmol min}^{-1} (\text{mg dry wt cells})^{-1}$] as a result of other metabolic processes including N_2 -fixation, photorespiration and protein turnover. At lower concentrations of urea the effect of methionine sulfoximine was much more significant, presumably because the rate of incorporation of NH_3 by glutamine synthetase is a greater proportion of the urease rate at the lower urea concentration, and possibly also because urea uptake becomes rate-limiting at lower concentrations.

Whole-cell measurements were used to assess the dependence of urease activity on urea concentration: the apparent K_m was not significantly different from that measured in cell-free extracts. A urea concentration of 10 mM was sufficient to saturate the enzyme in both cases.

In summary, we have shown that *A. cylindrica* has a constitutive nickel-dependent urease. However, cells will grow efficiently in the total absence of nickel (Daday *et al.*, 1985) and thus the precise role of urease in the nitrogen metabolism of this organism remains to be elucidated.

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