Short Communication

Effect of Urea on Ammonium-dependent Synthesis of Carbamyl Phosphate during Spore Germination of Geotrichum candidum

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Received for publication December 27, 1971

ABSTRACT

The specific activities of enzymes catalyzing the ammonium-dependent carbamyl phosphate synthesis (NH$_4$-CPS) and the glutamine-dependent carbamyl phosphate synthesis (GLN-CPS) were increased during germination by approximately 5- and 1.7-fold respectively in the presence of 35 mM urea. The increase of NH$_4$-CPS and GLN-CPS levels occurred immediately after the onset of germination and prior to the appearance of germ tube. Ammonium also stimulated the NH$_4$-CPS activity, but the induction caused by urea was about three times higher than that by ammonium.

Both NH$_4$-CPS and GLN-CPS were highly labile. NH$_4$-CPS was obtained free of GLN-CPS after (NH$_4$)$_2$SO$_4$ fractionation and diethylaminoethyl cellulose chromatography. The optimum pH for NH$_4$-CPS was 8.5 as opposed to broad pH optimum of pH 5.6-8 for the reverse reaction. The Km values obtained for NH$_4$, glutamine, and carbamyl phosphate were 12 mM, 0.5 mM, and 0.083 mM, respectively.

Carbamyl phosphate is a precursor for metabolic pathways leading to pyrimidine nucleotides and to arginine (8). Enzymes have been found in microorganisms and higher plants which synthesize CAP$^1$ from ATP, HCO$_3^-$, and glutamine or NH$_4$. The first type is carbamate kinase (ATP: carbamate phosphotransferase, EC 2.7.2.2) which uses only NH$_4$ as its nitrogen source. Carbamate kinase probably functions as a catabolic enzyme under in vivo conditions (9). The second enzyme type is classified as CAP synthetase showing preference for glutamine over NH$_4$ as a nitrogen donor (13). The glutamine-dependent enzyme apparently possesses an anabolic role (5, 15).

The presence of both carbamate kinase and CAP synthetase in Bacillus subtilis has been recently reported (7). Fungi appear to have two CAP synthetases; one, specific for pyrimidine synthesis, is controlled through repression and end product inhibition by the pyrimidine pool (11, 12, 19), and one, specific for arginine, is repressed and inhibited by arginine (5, 11).

Spores of Geotrichum candidum lack urease, yet urea is readily metabolized during germination by means of ATP and urea amidolyase (18). Previous studies have shown that addition of urea to the germinating medium of G. candidum results in derepression of ornithine transcarbamylase (2). The present work describes the effect of urea on the NH$_4$-dependent synthesis of CAP in relation to the possible physiological function of the latter reaction in G. candidum.

MATERIALS AND METHODS

Procedures for obtaining and germinating conidia of G. candidum were described (1). The germination medium (YEG) contained 0.1% yeast extract, 0.5% glucose, and 50 mM phosphate buffer, pH 7.5. The germinated spores were harvested (18) and either used immediately for determination of endogenous ammonia or maintained frozen until used for preparation of cell-free extracts. Enzyme extraction was performed according to Barash and Mor (2) in 100 mM tris-HCl buffer, pH 7.8, which contained 20 mM MgCl$_2$, 10 mM 2-mercaptoethanol, and 10% v/v glycerol. Enzyme assays in the crude extracts were carried out immediately following dialysis of 3 hr against the extraction buffer.

When enzyme fractionation was required, the crude extract was centrifuged at 105,000 g for 45 min, and the enzyme was precipitated by ammonium sulfate between 25 and 50% saturation. The precipitate was dissolved in 5 to 10 ml of the extraction buffer and dialyzed against the same buffer for 3 hr. The enzyme preparation was then placed on a column (2 x 15 cm) of DEAE-cellulose (Whatman microgranular DE-52) previously equilibrated with 50 mM tris-HCl, pH 8.0, containing 10 mM 2-mercaptoethanol, 20 mM MgCl$_2$, and 10% v/v glycerol. Enzyme elution was carried out by linear increase in KCl concentration from 0 to 0.5 M.

The enzymatic synthesis of CAP was determined by both colorimetric and radioactive methods. The assay system for NH$_4$-dependent CAP synthesis (NH$_4$-CPS), using the colorimetric method, contained (in a total volume of 1.5 ml): ATP, 10 $\mu$moles; MgCl$_2$, 20 $\mu$moles; tris-HCl buffer, pH 8.5, 100 $\mu$moles; (NH$_4$)$_2$CO$_3$, 100 $\mu$moles; and 0.2 ml of enzyme preparation. For the glutamine-dependent reaction (GLN-CPS), the (NH$_4$)$_2$CO$_3$ was replaced by 100 $\mu$moles of NaHCO$_3$, and 100 $\mu$moles of glutamine. A reaction mixture without ATP served as a control. After incubation for 20 min at 30°C, the reaction was stopped by adding 0.5 ml of 2 N HCl and then boiling for 10 min. The last treatment resulted in quantitative transformation of CAP into urea (20). The reaction mixture was brought to 5% with respect to perchloric acid, and then cooled to 4°C. After centrifugation for removal of proteins, urea was estimated by the colorimetric method of Hunninghake and Grisolia (6). One unit of activity is defined as the amount of enzyme that catalyzes the synthesis of 1 $\mu$ mole CAP/hr. Results presented are an average of at least four different experi-

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1 Abbreviations: CAP: carbamyl phosphate; DEAE: diethylaminoethyl.
ments. In the radioactive method, enzyme activity was determined by converting CAP (formed from ¹³C-labeled bicarbonate) to acid-stable citrulline (4). The enzymatic synthesis of ATP from ADP and CAP was carried out at 30 C. The reaction mixture contained (in a total volume of 1 ml): tris-HCl buffer, pH 7.6, 100 μmoles; ADP, 10 μmoles; CAP, 10 μmoles; MgCl₂, 20 μmoles and enzyme preparation. The reaction was stopped after 20 min by adding 0.2 ml of 1 N HCl and the acidified solution was cooled to 0 C and neutralized after 10 min by adding 0.3 ml of 1 M tris. The ATP concentration in 0.5 ml of the reaction mixture was determined enzymatically according to Kornberg (10). Acetyl phosphokinase activity was measured by the production of acetyl-P as described by Rose et al. (16).

Protein was determined by the method of Lowry et al. (14). Free ammonium was measured in the crude cell-free extract according to Brown et al. (3). ATP, ADP, and lithium CAP were obtained from Fluka, Switzerland. Hexokinase (crystal- line suspension) was obtained from Boehringer Mannheim Company and NaH¹³CO₃ was bought from Radiochemical Centre, Amersham, England. All other chemicals used were of analytical grade.

RESULTS AND DISCUSSION

Results in Figure 1 show that the specific activities of both, NH₃-CPS and GLN-CPS were increased proportionally to urea concentration until about 35 mM urea was reached. Higher urea concentrations showed a decrease in both enzyme activities which might result from inhibitory levels of intracellular urea (18). However, whereas the specific activity of NH₃-CPS increased 4.5-5 fold in the presence of 35 mM urea, the increase in GLN-CPS was less than 2-fold (Fig. 1). The preferential induction of NH₃-CPS activity by urea increased the NH₃-CPS/GLN-CPS ratio from approximately 0.6 to 1.9. Similar results were obtained when the enzyme activities were measured with the radioactive instead of the colorimetric procedure. Acetyl phosphokinase, which possesses carbamate phosphorylating activity and can utilize NH₃ as a nitrogen donor (20), was not detected in cell-free extracts of G. candidum.

The stimulation of NH₃-CPS and GLN-CPS levels by urea was studied as a function of the germination process (Fig. 2). Addition of 35 mM urea to YEG did not affect the rate of germination. The preferential increase in level of NH₃-CPS took place immediately following the onset of incubation and

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**Table I. Effect of Ammonium on Activities of NH₃-CPS and GLN-CPS**

<table>
<thead>
<tr>
<th>Addition to Germination Medium</th>
<th>NH₃-CPS</th>
<th>GLN-CPS</th>
<th>NH₃-CPS/GLN-CPS</th>
<th>Intracellular Ammonia</th>
</tr>
</thead>
<tbody>
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<td>mM</td>
<td>units/mg</td>
<td>protein</td>
<td>ratio</td>
<td>μmoles/mg protein</td>
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<td>0.60</td>
<td>0.019</td>
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<td>0.39</td>
<td>0.64</td>
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<tr>
<td>NaHCO₃, 10</td>
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<td>0.41</td>
<td>0.88</td>
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<tr>
<td>(NH₄)₂CO₃, 10</td>
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<td>0.32</td>
<td>0.68</td>
<td>0.068</td>
</tr>
<tr>
<td>Urea, 35</td>
<td>0.39</td>
<td>0.41</td>
<td>0.95</td>
<td>0.027</td>
</tr>
</tbody>
</table>

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**Fig. 1. Effect of urea concentration on the specific activities of NH₃-CPS and GLN-CPS.** Spores were germinated in YEG medium supplemented with increasing concentrations of urea. ○: NH₃-CPS; △: GLN-CPS.

**Fig. 2. Effect of urea on the levels of NH₃-CPS and GLN-CPS during spore germination.** Spores were germinated in YEG medium with 0.035 mM urea or without urea. A: Influence of urea on germination rate. Spores were germinated in: (×) YEG; (□) YEG + urea. B: Influence of urea on enzyme activities. ○: NH₃-CPS in YEG; ●: NH₃-CPS in YEG + urea; △: GLN-CPS in YEG; ▲: GLN-CPS in YEG + urea.
prior to the appearance of germ tube. The level of NH$_2$-CPS reached a maximum after 3 hr when about 90% of the spores showed germ tube protrusion.

Further experiments were aimed at determining whether the increase in enzyme activities is dependent upon protein synthesis rather than upon activation of pre-existing enzymes. Germinating spores of G. candidum were insensitive to available specific protein synthesis inhibitors. Since a high urea concentration (0.3 M) and sodium azide (1 mM) inhibit protein synthesis in G. candidum (1, 18), the effect of these compounds on induction of NH$_2$-CPS and GLN-CPS was determined. Addition of either one of the latter compounds during germination prevents any increase in CPS activities in spite of an elevation in endogenous urea. Urea itself (up to a final concentration of 0.2 M) had no effect on the CPS activities on preincubation with crude cell-free extracts. The results may support an assumption that the urea effect is at the de novo synthesis rather than enzyme activation level.

The urea concentration required for induction is high (Fig. 1), and it might be questionable whether it serves as the inducer itself or its precursor. The presence of ATP-dependent urea amidolysase in G. candidum spores (18) raised the possibility that the preferential increase of NH$_2$-CPS activity is caused by the breakdown products of urea. Data given in Table 1 show that ammonium increased the specific activity of NH$_2$-CPS by about 2-fold, as compared to almost 5-fold by urea. The activity of GLN-CPS was also slightly affected by ammonia but the NH$_2$-CPS/GLN-CPS ratio in the presence of urea nearly doubled that of ammonia. Attempts to demonstrate correlation between different ammonia concentrations and induction of CPS activities gave negative results. However, low concentration of intracellular ammonium, as achieved by addition of glutamate, did not affect CPS activities (Table I). Since spores germinated with 35 mM urea contain even less endogenous ammonia than spores germinated with glutamate, the urea effect might not be explained merely on the basis of intracellular levels of free ammonia. These findings could lead to an assumption that the nitrogen obtained from the enzymatic breakdown of urea possesses a unique ability to induce CPS activities as compared to free ammonia. A similar peculiar urea effect on regulation of glutamate dehydrogenases was observed by Sanwal and Lata (17) in Neurospora.

If both CPS activities were performed by the same enzyme, the level of GLN-CPS would increase to correspond with the increase in the level of NH$_2$-CPS. Therefore, the preferential stimulation of NH$_2$-CPS activity by urea suggests that the two activities might be associated with different proteins. Attempts to separate the ammonium- and glutamine-dependent reactions by (NH$_4$)$_2$SO$_4$, as was achieved in Neurospora (5), were unsuccessful. Both activities were precipitated between 25 and 50% saturation, and the NH$_2$-CPS/GLN-CPS ratio was similar to that in the crude extract. Fractionation was handicapped by the high lability of the enzyme activities. NH$_2$-CPS was slightly more stable than GLN-CPS. The CPS activities were stabilized to some extent by addition of 10% glycerol; however, both activities were constantly reduced and disappeared within 3 to 4 days at 0 C. The maximal purifications obtained from NH$_2$-CPS after (NH$_4$)$_2$SO$_4$ and DEAE steps were 2- and 5-fold, respectively. Although a NH$_2$-CPS preparation was obtained without traces of GLN-CPS activity by chromatography on DEAE-cellulose, the GLN-CPS could not be recovered after the column fractionation. Therefore, the possibility that the lack of GLN-CPS activity in the NH$_2$-CPS preparation results from conformational changes in the active site of the enzyme rather than a true separation of a NH$_3$-specific enzyme cannot be excluded.

The pH optimum for NH$_2$-CPS was 8.5. The enzymatic activity for the synthesis of ATP from CAP and ADP had a very broad pH optimum from pH 5.6 to pH 8.0. The $K_m$ values extrapolated from Lineweaver-Burk plots for NH$_3$, glutamine, and CAP were 12 mM, 0.5 mM, and 0.083 mM, respectively.

The present results may suggest that the breakdown of urea in G. candidum by urea amidolysase is associated with an increase in CAP formation or utilization. Perhaps the physiological function of the NH$_2$-CPS activity is in the efficient conversion of urea-nitrates to citrulline via CAP formation or in the utilization of CAP derived from citrulline phosphorylase (2) to ATP and NH$_3$. This premise is upheld by the observations that urea increases the specific activity of ornithine transcarbamylase (2) and that urea-$^{14}$C is preferentially incorporated into citrulline (18).

LITERATURE CITED