Urea Carboxylase and Allophanate Hydrolase

TWO COMPONENTS OF ADENOSINE TRIPHOSPHATE:UREA AMIDO-LYASE IN SACCHAROMYCES CEREVISIAE*

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SUMMARY

The ATP:urea amidolyase reaction of yeast has been shown to consist of two distinct activities, an avidin-sensitive, thiourea-insensitive urea carboxylase and an avidin-insensitive, thiourea-sensitive allophanate hydrolase. Mutants lacking each of these activities have been isolated and were found to complement each other.

Enzyme preparations and assay procedures were similar to those described previously (1). The specific activities of the preparations used in this work ranged from 0.2 to 0.6 and 1.2 to 1.6 units per mg for urea-dependent ADP production and allophanate-dependent CO₂ production, respectively.

M-25, the wild type strain employed, is the prototrophic diploid resulting from the cross, XT1172-S185 (α, ade, le) × XT1172-S62 (α, his, le, un). A series of mutants unable to grow on urea as the sole nitrogen source were isolated following EMS mutation (8) of XT1172-S185. The mutagenized culture was diluted and plated to give approximately 100 colonies per plate on YEPD agar (9) containing 0.1% (NH₄)₂SO₄. After 2 days at 30°, each plate was replicated to a series of four minimal (10) plates containing 2% glucose, 120 μg per ml of leucine, 20 μg per ml of adenine, and a nitrogen source to screen for those colonies specifically deficient in urea metabolism. Nitrogen sources for the four types of plates were 1.0 × 10⁻² M urea, 0.1 % (NH₄)₂SO₄, 3.7 × 10⁻³ M arginine, and 7.5 × 10⁻³ M ornithine, respectively. Purification of mutants, complementation tests, and construction of prototrophic diploids carrying the various mutant alleles were performed employing standard genetic methods (9). M-62 and M-64 are prototrophic diploids homozygous for mutant alleles E-145 and E-142, respectively.

Growth of the mutants for determination of the enzymes was accomplished on minimal medium containing 0.6% glucose and 0.02% (NH₄)₂SO₄. Urea, 1.0 × 10⁻⁴ M, was added at a cell density of approximately 6 × 10⁶ cells per ml and after one generation of growth the cultures were made 10 μg per ml in cycloheximide and the cells were collected by centrifugation. After resuspension in 2.5 ml of 0.05 M Tris, pH 7.9, containing 5% glycerol, 2.0 × 10⁻⁴ M EDTA, and 3.0 × 10⁻² M mercaptoethanol, the cells were permeabilized with the method of Ramos et al. (11), with the exception that the procedures were carried out at 0°.

Synthesis of [¹⁴C]Allophanate (12)—[¹⁴C]Urea, 2.0 × 10⁻² mole and 1 mCi, was placed in a 20-ml round bottom flask and the ethanol (radioactive urea is commercially available as an ethanol solution) removed under a gentle stream of dry air. To the urea was added 1.0 × 10⁻⁴ mole of methyl chloroformate. The mixture was refluxed for 3 hours at 66°. The reaction was then terminated by cooling and addition of distilled water. [¹⁴C]-
Table I
Cofactor requirements of urea degradation system

Production of $^{14}$CO$_2$ from $[^{14}$C]urea. The complete reaction mixture contained in a volume of 1.0 ml: 0.1 M Tris, pH 7.9, 80 mM KCl, 5 mM MgSO$_4$, 2 mM urea (specific activity of $7.0 \times 10^6$ cpm per pmole), 4 mM KHCO$_3$, 2 mM ATP, and 100 µg of protein. The reactions were terminated after 15 min at 30°C and $^{14}$CO$_2$ was assayed as previously described (1). Urea-dependent ADP production was monitored by coupling the ADP-generating system to pyruvate kinase and lactic dehydrogenase. NADH oxidation was then monitored at 340 nm. The complete reaction mixture contained in a volume of 1.0 ml: 0.1 M Tris, pH 7.9, 80 mM KCl, 5 mM MgSO$_4$, 2 mM ATP, 2 mM urea, 5 mM dithiothreitol, 65 mM KHCO$_3$, 0.6 mM NADH, 4 mM P-enolpyruvate, 19 µg of pyruvate kinase, 68 µg of lactic dehydrogenase, and 217 µg of DEAE-purified protein. The assay was conducted at 23°C. $^{14}$CO$_2$ from $[\text{ureido-}^{14}$C]allophanate. The complete reaction mixture contained in a volume of 1.0 ml: 0.1 M Tris, pH 7.9, 80 mM KCl, 5 mM MgSO$_4$, 5 mM dithiothreitol, 4 mM potassium allophanate (specific activity of $7.0 \times 10^4$ cpm per pmole) and 104 µg of DEAE-purified protein. The reaction was terminated after 10 min at 30°C and $^{14}$CO$_2$ was assayed as described previously (1).

<table>
<thead>
<tr>
<th>Component omitted</th>
<th>Amount $^{14}$CO$_2$ from $[^{14}$C]urea</th>
<th>Amount of ADP produced per min</th>
<th>Amount $^{14}$CO$_2$ from $[\text{ureido-}^{14}$C]allophanate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>50,211</td>
<td>41.2</td>
<td>116,549</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K$^+$</td>
<td>22,650</td>
<td>40.7</td>
<td>124,318**</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>375</td>
<td>0.6</td>
<td>114,858</td>
</tr>
<tr>
<td>ATP</td>
<td>187</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KHCO$_3$</td>
<td>9,090</td>
<td>10.1</td>
<td></td>
</tr>
<tr>
<td>Enzyme protein</td>
<td>124</td>
<td></td>
<td>230</td>
</tr>
<tr>
<td>Urea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None + 100 µg avidin$^b$</td>
<td>135</td>
<td>3.9</td>
<td>119,818</td>
</tr>
</tbody>
</table>

$^a$ It should be noted that the enzyme preparation used contained 0.15 M KCl so that the final KCl concentration in the reaction mixture was $2.0 \times 10^{-2}$ M.

$^b$ The enzyme preparations were previously incubated with the avidin for 10 to 20 min before initiation of the reaction.

Allophanate methyl ester was collected from the ice-cold mixture by filtration. This ester was then dissolved in 5.0 ml of 0.5 M KOH and the solution was incubated at 37°C for 14 hours during which time it was saponified. At the conclusion of the incubation period the mixture was cooled to 4°C and 5 volumes of absolute ethanol and 1 volume of dry ethyl ether were added. During an overnight incubation of the resulting solution at 4°C the potassium allophanate crystallized out of solution and was collected the next morning by filtration. Due to the lability of allophanate in solutions with a pH below 7.0 the dry compound was always dissolved in 0.01 M Tris buffer, pH 7.9.

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**Fig. 1.** Effect of thiourea on urea-dependent ADP production (A) and $[^{14}$C]urea-dependent $^{14}$CO$_2$ production (B). The reaction mixtures were similar to those described in Table I except that in A 21 µg of protein were used and in B 31 µg were used. An equal amount of ethanol (20 µl) was substituted for the thiourea (dissolved in 20 µl of ethanol) when this effector was omitted.
Fig. 3. Theoretical effect of allophanate addition upon [14C]-urea-dependent 14CO2 production. It was assumed that: (a) the production of allophanate from urea is a zero order reaction with a rate constant of 0.034 μmole per min per ml and (b) the production of CO2 from allophanate follows Michaelis kinetics with V_max = 0.15 μmole per min per ml and K_m = 0.37 mM. The resulting differential equations were numerically integrated.

Fig. 4. Effect of allophanate addition on urea-dependent ADP (A) and 14CO2 production (B). The assay procedures were similar to those in Table I. Urea had a specific activity of 1.4 x 10^6 cpm per μmole. Micrograms (61) of protein were used in each 1.0-ml reaction mixture.

Fig. 5. Complementation of mutants unable to utilize urea as the sole nitrogen source. The mutants, isolated as described under "Materials and Methods," contained the lel, ad6 and α mating type markers of their parent. Mating with XTI172-S62 allowed the construction of a series of strains, each carrying a different mutant allele as well as the his and ur markers and an α mating type. These a and α strains were mated pairwise and the resulting prototrophic diploids were tested for their ability to grow on minimal plates containing 2% glucose and 1.0 x 10^-3 M urea as the only nitrogen source. (+) indicates growth and (−) indicates no growth.

Fig. 6. Production of 14CO2 from [14C]urea in mutant strains M-62 and M-64 were grown and permeabilized cell suspensions prepared as described under "Materials and Methods." Assay conditions were similar to those in the [14C]urea-dependent 14CO2 production assay of Table I; each 1.0-ml reaction mixture contained 0.15 ml of cell suspension or in the case of the mixed suspensions, 0.15 ml of each cell suspension.

RESULTS

Three principal assay methods (Table I) were used to dissect the urea degradation system: (a) the release of 14CO2 from [14C]-urea, (b) the urea-dependent production of ADP, and (c) the cleavage of [ureido-[14C]]allophanate yielding 14CO2. As shown in Table I, the reactions assayed by the first two methods require ATP, HCO_3^-, and Mg^2+ and are sensitive to avidin whereas the
reaction measured by the third method requires only the substrate, allophanate, and is totally insensitive to avidin. It is of interest that all of the partial reactions of other biotin carboxylases are fully sensitive to avidin (4-7).

To ascertain the possible existence of multiple, nonidentical urea- and allophanate-binding sites, the effect of thiourea on the enzymatic degradation of urea was investigated. As shown in Fig. 1, the presence of this analogue has no significant effect upon urea-dependent ADP production, but inhibits the release of \(^{14}\)CO\(_2\) from \(^{14}\)Curea approximately 90%. In addition it has been shown\(^2\) that thiourea will not support the production of ADP and, therefore, cannot substitute for urea in the carboxylation reaction. These results suggest that thiourea inhibition of the total reaction is probably due to inhibition of an activity subsequent to the carboxylation of urea. This indicates that there are two sites which bind allophanate (Reactions 2 and 3). Further, these sites must be separated to the extent that the inactivation (by either avidin or thiourea) of one site has no effect upon the other.

If Reactions 2 and 3 are catalyzed by separate proteins, it is reasonable to inquire whether or not these proteins have different heat sensitivities. The results illustrated in Fig. 2 show that urea-dependent ADP production is indeed sensitive to heat under conditions where allophanate cleavage is relatively insensitive.

\(^{1}\) P. A. Whitney and T. G. Cooper, in preparation.

\(^2\) P. A. Whitney and T. G. Cooper, in preparation.
The existence of two tandem reactions (Reactions 2 and 3) would necessitate enzymatically produced allophanate to be in rapid equilibrium with allophanate added to the reaction medium. On this assumption, it is possible to calculate the effect of added nonradioactive allophanate upon $^{14}$CO$_2$ accumulation from $[^{14}C]$urea. The results of these calculations, presented in Fig. 3, show that the addition of allophanate should result in a greatly increased lag in radioactive product accumulation; if enzymatically synthesized allophanate remained tightly bound to the enzyme, no large effect would be expected. The actual production of $^{14}$CO$_2$ from $[^{14}C]$urea and urea-dependent production of ADP, in the presence and absence of added allophanate, is shown in Fig. 4. The behavior observed is qualitatively that which would be expected if the intermediate in a set of tandem reactions was undergoing isotopic dilution (see Fig. 3).

In addition to these results which define two activities composing the urea degradation system, two activities have been distinguished genetically. Mutants unable to use urea as sole nitrogen source were isolated and divided into two groups on the basis of complementation (Fig. 5): diploids heterozygous for mutant alleles belonging to different groups could grow on urea, while diploids heterozygous for two alleles from the same group could not. Preliminary evidence indicates that these two complementation groups are linked (14 parental ditypes out of 14 tetrads (13)). One representative mutant from each group was grown on ammonia with urea added as an inducer and "permeabilized" cells (see "Materials and Methods") were prepared. As shown in Fig. 6 neither of these preparations was able to carry out the production of $^{14}$CO$_2$ from $[^{14}C]$urea, but a mixture of the two permeabilized cell suspensions degraded $[^{14}C]$urea to $^{14}$CO$_2$. The data in Fig. 7 show that one of these mutant strains (M-62) was able to cleave allophanate, while, as shown in Fig. 8, the other mutant strain (M-64) was able to accumulate $[^{14}C]$allophanate from $[^{14}C]$urea when provided with the appropriate carboxylation cofactors. These are the expected results if it is assumed that separate polypeptides are responsible for the synthesis and degradation of allophanate.

**DISCUSSION**

Our results show that the catabolism of urea involves two enzymatic activities, a urea carboxylase (urea:CO$_2$ ligase (ADP) 6.3.4.6) and an allophanate hydrolase (allophanate amidohydrolase 3.5.1.13) catalyzing Reactions 2 and 3, respectively. Comparing the relative amounts of urea carboxylase (0.35 unit) and allophanate hydrolase (1.37 units) in a partially purified enzyme preparation, a 4-fold excess of the hydrolase is observed. This excess, the lack of cofactor requirements for the hydrolase and an apparent Michaelis constant of allophanate hydrolase for allophanate of 0.37 mM makes it unlikely that allophanate would ever appear as an intermediate except in trace amounts. This may explain why these reactions were not separated by Roon and Levenberg (14) during their early studies on the stoichiometry of the ATP:urea amidohydrolase reaction.

In as much as the urea carboxylase and allophanate hydrolase activities have been shown to be separable by a number of criteria it is important to ask whether these are the activities: (a) of a single protein, (b) of the components of a multiprotein complex, or (c) of two separate proteins. In other fungal systems enzymes in a pathway whose structural genes are linked are usually found as enzyme complexes (15-17). Limited support for this possibility is afforded by our inability to physically separate these activities through the preliminary application of a number of techniques (ammonium sulfate fractionation, DEAE-cellulose and brushite columns eluted with linear salt gradients, and glycerc gradient centrifugation). If in fact these activities do form a multienzyme complex in yeast they would then contrast with the recent report from Thompson and Muenster (18) that urea carboxylase and allophanate hydrolase from *Chlorella* can be separated on brushite columns by a stepped gradient.

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**REFERENCES**

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*P. A. Whitney and T. G. Cooper, unpublished observations.*
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