Urea Carboxylase from Saccharomyces cerevisiae

EVIDENCE FOR A MINIMAL TWO-STEP REACTION SEQUENCE*

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SUMMARY

Urea carboxylase, an avidin-sensitive enzyme catalyzing the ATP-dependent carboxylation of urea, has been shown to catalyze this reaction in a sequence of at least two steps. In the presence of all of the required carboxylation cofactors except urea, an enzyme-CO$_2$ complex is formed. This complex was isolated by Sephadex chromatography and was capable, in the absence of added cofactors, of transferring the bound CO$_2$ to urea. In addition to the carboxylation of urea this protein was capable of carboxylating free d-biotin, as well as the urea analogues formamide, formylurea, and acetamide. These data are consistent with the suggestion that urea carboxylase follows a reaction mechanism similar to those of the more thoroughly studied propionyl-CoA, acetyl-CoA, and pyruvate carboxylases.

Long before the function of biotin in carboxylation reactions had been elucidated, DiCarlo et al. reported (1) that this vitamin was required in large quantities for the growth of Saccharomyces cerevisiae on certain nitrogenous compounds. These compounds included urea, allantoic acid, allantoic acid, and hydroxamic acid. A possible explanation for this observation was found in Levenberg's demonstration that a urea-degradative activity solubilized from Chlorella and Candida utilis was sensitive to avidin. This activity, urea amidolyase, was shown by Roon and Levenberg (2) to catalyze the reaction

$$
\text{Urea} + \text{ATP} \rightarrow \text{allophanate} + \text{ADP} + \text{Pi}
$$

The avidin inhibition of this activity suggested that biotin was functioning in the reaction and prompted Whitney and Cooper (3) to investigate the possible involvement of biocarboxylate as a cofactor of the reaction. Their results demonstrated that the reaction possessed an absolute requirement for bicarbonate and led to the suggestion that Reaction 1 should be written

$$
\text{Urea} + \text{ATP} + \text{HCO}_3^- \rightarrow \text{CO}_2 + 2\text{NH}_3 + \text{ADP} + \text{Pi}
$$

These investigators have recently shown that in Saccharomyces the urea amidolyase reaction (Reaction 2) is in fact the sum of two reactions,

$$
\text{Urea} + \text{ATP} + \text{HCO}_3^- \rightarrow \text{allophanate} + \text{ADP} + \text{Pi}
$$

$$
\text{Allophanate} \rightarrow \text{CO}_2 + 2\text{NH}_3
$$

catalyzed by urea carboxylase and allophanate hydrolase, respectively (4). The proposal that two separate activities are responsible for the degradation of urea in yeast is in agreement with a preliminary report by Thompson and Muenster (5); these investigators found that in Chlorella vulgaris the activities catalyzing Reactions 3 and 4 could be separated on brushite. The finding that urea is degraded in two sequential steps (the carboxylation of urea and the hydrolysis of allophanate) greatly simplifies the mechanisms that may be suggested to explain the avidin-sensitive reaction and raises the question of whether or not urea is carboxylated according to the mechanism followed by other, more thoroughly studied, biotin-dependent carboxylases.

From investigations in a number of laboratories (6-13), a reasonably complete picture of the mechanism of biotin-dependent carboxylases has emerged. Using a number of different biotin-containing carboxylases, these investigators have shown (Fig. 1) that biotin-dependent carboxylations are catalyzed in at least two steps. The first step (Reaction 5) is the ATP, Mg$^{2+}$-dependent carboxylation of position 1'-N of the biotin prosthetic group. The second reaction is the cofactor-independent transcarboxylation of the activated carbonyl group to an appropriate acceptor (Reaction 6). If urea carboxylase is catalyzing Reaction 3 according to this type of mechanism, then it should be possible to demonstrate these half-reactions (Reactions 5 and 6) in a manner similar to that previously used in the studies of 3-methylcrotonyl-CoA, acetyl-CoA, and propionyl-CoA carboxylases. For these carboxylases, it was possible to isolate (10, 11, 14) the enzyme-bound CO$_2$ intermediate hypothesized in Reaction 5 and then demonstrate that...
E-biotin + ATP + HCO₂⁻ Mg⁺⁺ → ADP + P₁ + E-biotin ~ CO₂

E-biotin ~ CO₂ + acceptor = E-biotin + carboxylated acceptor

**Fig. 1.** Reaction sequence followed by biotin-dependent acetyl-CoA carboxylase.

this bound, activated "CO₂" could be transferred to an appropriate acceptor molecule. The data presented here are the results of such experiments and support the contention that the mechanism of the urea carboxylase reaction is similar to that of other biotin-dependent carboxylases.

**METHODS**

**Enzyme Purification**—The enzyme preparations used in the present studies were partially purified using the procedures described by Whitney and Cooper (15). Unless otherwise indicated, the protein employed was that yielded from the DEAE-cellulose chromatography step of the purification scheme.

**"Permeabilized" Cells**—Whole cell preparations for the determination of any of the activities associated with the carboxylation of urea were made using a "permeabilization" technique similar to that of Ramos et al. (16). Ten-milliliter portions of the cultures were sampled into chilled tubes containing sufficient cycloheximide to give a concentration of 10 μg per ml. The cells were collected by centrifugation and resuspended in 0.5 ml of 0.05 M Tris buffer, pH 7.9, containing 2 X 10⁻⁴ M EDTA, 2 X 10⁻⁴ M mercaptoethanol, and 5% glycerol. To this suspension were added 15 μl of a solution containing 3 mg of nystatin in 0.9 ml of methanol and 0.4 mg of cycloheximide in 0.1 ml of water.

**Urea-dependent Production of ADP**—ADP production was monitored spectrophotometrically by coupling the ADP-generating reaction to those of pyruvate kinase and lactic dehydrogenase. Concomitant NADH oxidation was determined at 340 nm. The complete reaction mixture contained in a volume of 1.0 ml: 100 mM Tris buffer, 80 mM KCl, 5 mM MgSO₄, 2 mM ATP, 2 mM urea, 5 mM dithiothreitol, 4 mM KHCO₃, 0.6 mM NADH, 4 mM P-enolpyruvate, 19 μg of pyruvate kinase, and 26 μg of lactic dehydrogenase. The final pH was 7.9.

**Isolation of [¹⁴C]Allophanate**—[¹⁴C]Allophanate was isolated using linear salt gradient elution from DEAE-cellulose. A washed DEAE-cellulose (Whatman DE-23) column (1.5 × 12 cm) was fully equilibrated with 0.01 M Tris hydrochloride buffer adjusted to pH 8.4. Following the application of the allophanate urea mixture, the column was washed with water and then eluted with a 200 ml, 0 to 1.0 M linear NaCl gradient. As shown in Fig. 3, the urea was quantitatively removed in the water wash while 0.15 M NaCl was required to elute the allophanate.

**Lactic Dehydrogenase**—Lactic dehydrogenase was assayed by monitoring NADH oxidation at 340 nm. The reaction mixture contained in a volume of 1.0 ml: 100 mM Tris buffer, pH 7.9, 5 mM potassium pyruvate, and 0.6 mM NADH.

**RESULTS**

To ascertain whether or not urea carboxylase was following the minimal two-step reaction sequence described above, an attempt was made to isolate the carboxylated enzyme intermediate. This was done by incubating a partially purified protein preparation with all of the carboxylation reaction cofactors except urea. After a 6-min incubation period, commercially obtained lactic dehydrogenase was added as a marker protein and the reaction mixture was chromatographed on Sephadex G-50. As shown in Fig. 3A, a considerable amount of radioactive CO₂ was excluded from the column coincident with the protein peak (lactic dehydrogenase activity). In order to eliminate the possibility that the CO₂ observed in these protein fractions was the result of nonspecific carbamate formation between CO₂ and available

**Fig. 2.** DEAE-cellulose chromatography of urea and allophanate. A mixture of [¹⁴C]urea and nonradioactive allophanate were chromatographed on DEAE-cellulose as described under "Methods." Urea was determined (●—●) by transferring a sample of each fraction into Aquasol and determining the amount of color formation at 420 nm. Ion strength (▲—▲) was measured through the use of a radiometer conductance meter.

**Fig. 3.** The isolation of [¹⁴CO₂]-enzyme. The enzyme preparation was a 35 to 45% ammonium sulfate fraction. The complete reaction mixture (A) contained in a volume of 0.7 ml: 143 mM Tris buffer, pH 7.9, 114 mM KCl, 7.1 mM MgSO₄, 7.1 mM ATP, 5.8 mM dithiothreitol, 5 μmoles and 122 μCi of [¹⁴C]KHCO₃, and 1.6 mg of enzyme protein. The reaction in B was identical with that in A except for the omission of ATP. Following a 6-min incubation at 0°, the reaction was terminated by the addition of 0.2 ml of 1 M Tris buffer, pH 8.4, 0.1 ml of 4% EDTA, and a small amount of the marker protein, lactic dehydrogenase. The entire mixture was immediately layered onto a Sephadex G-50 column (1.5 × 30 cm) equilibrated with 0.05 M Tris buffer, pH 8.4, containing 2 X 10⁻³ M mercaptoethanol. The column was eluted with the same buffer, and 1.5-ml fractions were collected. A 0.5-ml sample of each fraction was placed in a scintillation vial containing 0.1 ml of hyamine hydroxide, and the samples were counted in Aquasol. Lactic dehydrogenase was assayed as described under "Methods." The data are expressed as micromoles of NAD produced per min.
amino groups of the protein, the experiment was repeated in the absence of ATP, a required cofactor for the carboxylation of biotin (Reaction 5). As shown in Fig. 3A, the omission of ATP resulted in loss of 88% of the radioactivity from the protein peak. This suggests that only 12% of the radioactivity observed in the previous experiment was due to non-specific carbonate formation. If indeed a carboxylated biotin intermediate was formed in the above experiment, then it should be possible to transcarboxylate the bound $^{14}$CO$_2$ to the acceptor, urea, in the absence of any added cofactors. This was done using a protocol identical with that employed in Fig. 3A, with the exception that much more protein (20 mg) was used. After separation from the rest of the reaction mixture on Sephadex G-25, the $^{14}$CO$_2$-protein complex in a volume of 0.01 ml was incubated in the presence of 1.75 mM urea and 2 mM allophanate. At the conclusion of a 2-min incubation period at room temperature the transfer reaction was terminated by addition of 2 ml of absolute ethanol. Approximately 100 mg of nonradioactive carrier potassium allophanate was added and the allophanate crystallized as described earlier (4). The crystallized allophanate was dried, dissolved in 0.01 M Tris buffer adjusted to pH 7.9, and the solution was chromatographed on a DEAE-column (2.8 X 5 cm) prepared as described in Fig. 2. Fractions (3.5 ml) were collected and the linear salt gradient was initiated at Fraction 16. Each fraction was finally assayed for radioactivity and the presence of allophanate. (See Fig. 1 for methods used to determine allophanate concentration.)

It has been reported by Lynen and others that several of the urea carboxylases possess the ability to carboxylate free d-biotin as well as that covalently bound to the enzyme (7-9). Urea carboxylase was assayed for this activity and as shown in Fig. 5, the enzyme was capable of supporting biotin-dependent ADP production. These data offer indirect evidence that urea carboxylase also can catalyze the carboxylation of free d-biotin. Although the carboxylated product has not been conclusively identified as carboxybiotin, it has been possible to demonstrate the enzyme's ability to carboxylate free d-biotin. The demonstration (3) that the urea amidolyase reaction is autocatalytic with respect to bicarbonate, however, it can be suggested that the early specificity data indicate only that none of the compounds employed could be carboxylated and hydrolyzed. The compounds employed could be carboxylated and hydrolyzed.

In their early studies of the urea amidolyase reaction, Roon and Levenberg (20) presented data indicating that this reaction was absolutely specific for urea and that a variety of urea analogues neither activated nor inhibited the reaction. In view of the demonstration (9) that the urea amidolyase reaction is autocatalytic with respect to bicarbonate, however, it can be suggested that the early specificity data indicate only that none of the compounds employed could be carboxylated and hydrolyzed. Only compounds capable of participating in both reactions would have been recognized as alternative substrates since hydrolysis, and Levenberg (20) presented data indicating that this reaction was autocatalytic with respect to bicarbonate, however, it can be suggested that the early specificity data indicate only that none of the compounds employed could be carboxylated and hydrolyzed. Only compounds capable of participating in both reactions would have been recognized as alternative substrates since hydrolysis, with concomitant $^{14}$CO$_2$ production, would have been required to supply the bicarbonate requirement. The data in Fig. 8 demonstrate that several urea analogues can support ADP production if bicarbonate is supplied in the reaction mixture. The data in Table I summarize the results of this type of experiment performed on a wide variety of compounds and test (a) their inhibitory effects upon the combined urea carboxylase and allophanate hydrolase reactions (urea amidolyase activity) and
Fig. 6. Lineweaver-Burk plot of urea-dependent ADP production as a function of urea concentration (A) and as a function of ATP concentration (B). The spectrophotometric reaction was carried out as described under "Methods" except that urea (A) and ATP (B) were added at the indicated concentrations; each reaction mixture contained 117 μg of enzyme protein. V is expressed as micromoles per min.

Fig. 7. Lineweaver-Burk plot of urea-dependent ADP production as a function of HCO₃⁻ concentration. The spectrophotometric reaction was performed as described in Fig. 6 with 110 μg of protein. The values for "CO₂" concentration were corrected for "CO₂" contaminating the solutions by the methods described earlier (3). No corrections were made for the species of "CO₂" participating in the reaction since at the present time the identity of that species is not known.

(b) their ability to serve as acceptors in the transcarboxylation reaction (thus permitting ADP production in the absence of urea). From these data, it is clear that while only thiourea inhibits the over-all urea amidolyase reaction (by severely inhibiting the allopbanate hydrolase reaction), a number of analogues will serve as urea substitutes in the urea carboxylase reaction. Those analogues capable of supporting ADP production include formamide, acetamide, formylurea and N-methyl urea. In agreement with these data are the observations of Roon and Levenberg (20), who reported that acetamide and formamide will function as substrates for ADP production by the C. utilis enzyme.

These data demonstrate that the four analogues mentioned above may substitute for urea in the urea-dependent generation

<table>
<thead>
<tr>
<th>Analogue added</th>
<th>ADP (−Urea)</th>
<th>ADP (+Urea)</th>
<th>14CO₂ from [14C]urea</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.4</td>
<td>46.6</td>
<td>39,080</td>
</tr>
<tr>
<td>Allophanate</td>
<td>6.9</td>
<td>50.0</td>
<td>7,841</td>
</tr>
<tr>
<td>Thiourea</td>
<td>5.8</td>
<td>39.5</td>
<td>3,089</td>
</tr>
<tr>
<td>Pheny lurea</td>
<td>8.5</td>
<td>35.4</td>
<td>10,210</td>
</tr>
<tr>
<td>Formamide</td>
<td>20.3</td>
<td>46.6</td>
<td>52,478</td>
</tr>
<tr>
<td>Methylformamide</td>
<td>5.8</td>
<td>46.6</td>
<td>40,400</td>
</tr>
<tr>
<td>Acetamide</td>
<td>12.7</td>
<td>40.2</td>
<td>56,686</td>
</tr>
<tr>
<td>2-Chloroacetamide</td>
<td>5.8</td>
<td>46.6</td>
<td>52,134</td>
</tr>
<tr>
<td>Urethane</td>
<td>6.4</td>
<td>42.1</td>
<td>51,456</td>
</tr>
<tr>
<td>Formylurea</td>
<td>36.3</td>
<td>46.6</td>
<td>48,588</td>
</tr>
<tr>
<td>N-Methyl urea</td>
<td>11.6</td>
<td>45.1</td>
<td>38,200</td>
</tr>
<tr>
<td>N-Hydroxyurea</td>
<td>5.8</td>
<td>41.8</td>
<td>35,200</td>
</tr>
<tr>
<td>Hydantoic acid</td>
<td>5.0</td>
<td>61.2</td>
<td>60,217</td>
</tr>
</tbody>
</table>

* Thiourea was added as an ethanol solution; all other analogues were added as aqueous solutions. The final concentration of the analogues was 5 mM.
TABLE II
Cofactor requirements of urea carboxylase reaction

<table>
<thead>
<tr>
<th>Component omitted</th>
<th>Counts per min stable to gassing</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>$10^4$</td>
</tr>
<tr>
<td>None but with formylurea replacing formamide</td>
<td>23.7</td>
</tr>
<tr>
<td>ATP</td>
<td>61.0</td>
</tr>
<tr>
<td>Protein</td>
<td>11.5</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>9.5</td>
</tr>
<tr>
<td>Protein + Mg$^{2+}$</td>
<td>9.8</td>
</tr>
</tbody>
</table>

TABLE III
Effect of acid on methylated carboxylation product of urea carboxylase reaction

The conditions of the carboxylation reaction were identical with those described in Table II. The reaction was terminated by addition of 4 volumes of methanol and this was followed by the addition of sufficient 1 N diazomethane to give a persistent yellow color to the solution. The sample was then evaporated to a volume of 1.5 ml. A sample of each reaction mixture was taken for radioactivity measurement and an identical volume sample was added to 4 N acetic acid. After evaporating the acidified sample to dryness, 0.2 ml of water was added and the radioactivity contained in the sample was measured.

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Methylated product</th>
<th>Methylated product stable to mild acid treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formylurea</td>
<td>109.7</td>
<td>51.0</td>
</tr>
<tr>
<td>Formamide</td>
<td>88.6</td>
<td>43.8</td>
</tr>
</tbody>
</table>

The results summarized in Table II indicate that the urea carboxylase can participate in three reactions: (a) the ATP-dependent binding of CO$_2$ to the enzyme, (b) the ATP-dependent carboxylation of free d-biotin and (c) the cofactor-independent transcarboxylation of enzyme-bound CO$_2$ to urea. These results can be accounted for by suggesting that urea is carboxylated according to a two-step mechanism (Fig. 9) similar to those which have been suggested for other biotin-dependent carboxylases (10, 11, 21). Supporting this proposal is the observation that the carboxylation of urea is sensitive to avidin, a protein which binds specifically to biotin.

It is of interest that urea carboxylase appears to possess considerable flexibility in its substrate specificity. A number of urea analogues including acetamide, formamide, and formylurea were capable of supporting ATP cleavage. Through the use of radioactively labeled bicarbonate, it was possible to show that the latter two of these analogues were in fact carboxylated. This result is corroborated by the recent physiological experiments of Cooper and Whitney (22). Their experiments show that the actual inducer of the urea degradation system is most likely allophanate, the product of urea carboxylase. In addition they have shown that formamide will serve as a nonmetabolizable inducer in wild type strains, but will not perform this function in strains possessing a defective urea carboxylase. These urea carboxylase-defective strains presumably are not capable of carboxylating formamide to yield the allophanate analogue, N-carboxyformamide.

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REFERENCES
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