UREA CARBOXYLASE AND ALLOPHANATE HYDROLASE: TWO COMPONENTS OF A MULTIENZYME COMPLEX IN SACCHAROMYCES CEREVISIAE

P. A. Whitney[1] and T. G. Cooper

Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass. 02139 and Department of Biochemistry, University of Pittsburgh, Pittsburgh, Pa. 15213.

Received August 15, 1972

Summary: Saccharomyces cerevisiae can utilize urea as sole nitrogen source by virtue of its ability to degrade the compound to NH_3 and CO_2. Whitney and Cooper have shown that the conversion is accomplished in two enzymatic steps: (1) the carboxylation of urea yielding allophanate and (2) the hydrolysis of allophanate yielding NH_3 and CO_2. To determine whether these proteins compose a multienzyme complex, the response of urea carboxylase and allophanate hydrolase to several analytical separation and immunochemical precipitation techniques was monitored. The data obtained support the contention that urea carboxylase and allophanate hydrolase are components of a multienzyme complex.

Saccharomyces cerevisiae can utilize urea as a sole nitrogen source by virtue of its ability to degrade the compound to "CO_2" and ammonia. The overall reaction responsible for this degradation was shown by Roon and Levenberg (1) and Whitney and Cooper (2) to be

$$\text{Urea} + HCO_3^- + ATP \longrightarrow 2NH_3 + 2\ \text{"}CO_2\text{"} + ADP + P_i \qquad (1)$$

More recently, however, Whitney and Cooper (3) have presented evidence which indicate that the overall degradation in yeast is accomplished in two steps:

$$\text{Urea} + HCO_3^- + ATP \overset{Mg^{++}}{\rightleftharpoons} ADP + P_i + \text{Allophanate} \qquad (2)$$

$$\text{Allophanate} \longrightarrow 2NH_3 + 2\ \text{"}CO_2\text{"} \qquad (3)$$

which are catalyzed by a biotin-containing urea carboxylase and allophanate hydrolase respectively. They isolated mutant strains lacking each of these enzymatic activities and upon crossing the two types of mutant strains found no recombinants indicating that the presumed structural genes responsible for urea carboxylase and allophanate hydrolase are closely linked. In view of this observed linkage and the observations of Giles and others (4, 5) that the structural genes responsible for the synthesis of the components of a

multienzyme complex are clustered together, experiments were performed to determine whether or not urea carboxylase and allophanate hydrolase are the members of such a complex. The data presented here support the contention that the urea degradative enzymes are components of a multienzyme complex.

METHODS

Purification of urea carboxylase and allophanate hydrolase through ammonium sulfate precipitation was performed as described earlier (2); subsequent steps are described in the appropriate figure legends. Enzyme assay procedures were similar to those reported earlier (3). The reaction mixture, for the assay of alkaline phosphatase, contained in a volume of 1.0 ml: 100 mM Tris buffer, pH 7.9, 5 mM $MgCl_2$, 2 mM p-nitrophenyl phosphate, and 40 microliters of avidin-crude extract complex. The absorbance was monitored at 405 mμ.

RESULTS AND DISCUSSION

To determine whether or not urea carboxylase and allophanate hydrolase existed as an enzyme aggregate, the response of these two activities to several analytical separation techniques was monitored. As shown in the left panel of figure 1, chromatography of the 35 to 45% ammonium sulfate fraction on a brushite column using a linear salt (phosphate) gradient resulted in the elution of coincident peaks of urea carboxylase and allophanate hydrolase activities. This technique has recently been used to successfully separate these two activities in Chlorella (6). It is clear that in the case of the yeast enzymes, however, no separation was affected.

The 35 to 45% ammonium sulfate fraction was also applied to DEAE cellulose. As shown in the right panel of figure 1, elution of the protein with a linear KCl gradient also resulted in coincident peaks of the two activities. The fractions which contained the enzymatic activities were pooled, concentrated with carbowax, and layered onto a 10 to 30% sucrose gradient. The data in figure 2 indicate that urea carboxylase and allophanate hydrolase cosedimented on the sucrose gradient. The purification derived from these treatments have been summarized in Table I and attest the inability of these techniques to separate the two activities. The observation that the ratio of allophanate hydrolase to urea carboxylase (A/U column of Table I)

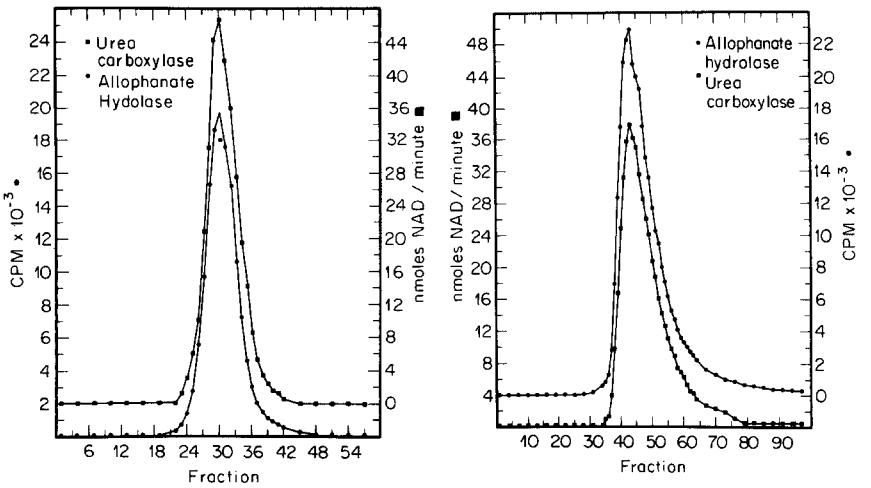

Figure 1. (left panel) Brushite chromatography of urea carboxylase and allophanate hydrolase. A 35-45% ammonium sulfate fraction was desalted and absorbed onto a 3.4 x 4.4 cm brushite column and the column was washed with 80 ml of the above buffer. The protein was then chromatographed using a 0.025-0.6M linear phosphate gradient at pH 7.0 and 2 ml fractions were collected. The total volume of the gradient was 300 ml. Fifty microliter aliquots of the fractions were assayed for urea-dependent ADP production and 15 microliter aliquots were assayed for ureido-[^{14}C] allophanate-dependent $^{14}CO_2$ production. For the radiochemical assay, each 1.0 ml reaction mixture contained 2.0 micromoles and 0.05 microcuries of ureido-[^{14}C] allophanate.
(right panel) DEAE-cellulose chromatography of urea carboxylase and allophanate hydrolase. A 35-45% ammonium sulfate fraction was desalted using G-25 and absorbed onto a 2.5 x 5.0 cm DEAE-cellulose column; the buffer used in this case contained 0.05M phosphate, pH 7.0, 25% glycerol, and 2 x 10^{-3}M mercaptoethanol. The chromatography on DEAE-cellulose was performed using a 200 ml, 0.0 to 0.5M linear KCl gradient; 1.4 ml fractions were collected. The fractions were assayed as described above.

did vary over a 1.7-fold range can be accounted for by the fact that these two activities have different sensitivities to heat (see figure 2 of 4) and, therefore, could also be expected to display different labilities to the various conditions employed in this purification scheme.

Although these data suggest that urea carboxylase and allophanate hydrolase are the activities of a single protein or of a multiprotein complex, the possibility that the apparent co-purification of the two activities was the result of the particular conditions and techniques employed can never be

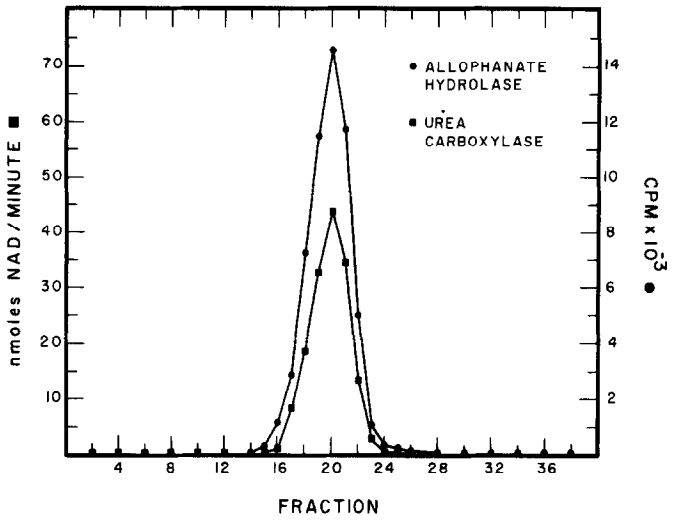

Figure 2. Sucrose gradient centrifugation of urea carboxylase and allophanate hydrolase. 0.25 ml of a solution containing concentrated DEAE-cellulose purified enzyme was diluted with 0.55 ml of 0.05 M phosphate buffer, pH 7.0, and layered onto a 10-30% sucrose gradient containing 0.05M phosphate buffer, pH 7.0, 0.1M KCl, and 2 x 10^{-3}M mercaptoethanol. The volume of the gradient was 11 ml. Centrifugation was at 4°C for 27.5 hours at 100,000 x g (33,000 rpm in an International Preparative Ultracentrifuge, Model B-60, SB-283 rotor). Forty-three fractions were collected and assayed as described in the legend to Figure 1.

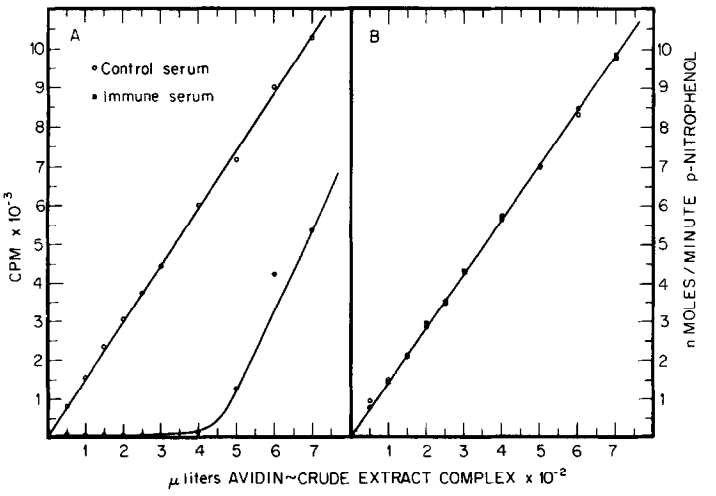

Fig. 3.

totally excluded. Therefore, this problem was addressed in another way which was predicated upon the facts that (1) avidin, a protein purified from egg whites, is capable of binding biotin in a kinetically irreversible manner (7) and (2) urea carboxylase is a biotin containing enzyme. A crude extract derived from wild type cells grown in the presence of urea was prepared and concentrated by precipitation with 70% ammonium sulfate; the resuspended protein was incubated with excess avidin. As shown in figure 3A, incubation of this preparation with immune serum (prepared against a homogeneous preparation of egg white avidin) resulted in the precipitation of the allophanate hydrolase activity, while incubation with control serum had no effect on this activity. In order to eliminate the possibility that this effect was due to non-specific protein precipitation, alkaline phosphatase activity was also monitored and the data in figure 3B demonstrate that this enzyme, in the presence of avidin, was not precipitated by either control or immune serum. This technique thus provides an independent method for distinguishing between two separate proteins and the components of a multiprotein complex or a single protein. The precipitation of allophanate hydrolase by antibody prepared specifically against avidin, which should bind specifically to the biotin containing urea carboxylase, is consistent with suggesting that urea carboxylase and allophanate hydrolase are the activities of a multiprotein complex.

Figure 3. Reaction of avidin specific antibody with avidin-crude extract complex. A crude extract was concentrated by the addition of solid ammonium sulfate to a final concentration of 70% saturation. The protein was resuspended and the resulting solution desalted by passage through a G-25 Sephadex column. Twelve ml of the desalted preparation, which contained 5.8 mg protein/ml, were incubated with 0.5 ml of 0.05M phosphate buffer, pH 7.0, containing 5.0 mg avidin/ml for 30 minutes at 22°C. A series of tubes was prepared which contained increasing amounts of this crude extract-avidin solution, 0.4 ml of immune serum or 0.4 ml of control serum, and 0.05M phosphate buffer, pH 7.0, to a final volume of 1.4 ml. After incubation overnight at 4°C, the precipitate was removed by centrifugation at 23,500 x g for 20 minutes. 200 microliters of the supernatant were assayed for ureido-[^{14}C] allophanate-dependent $^{14}CO_2$ production (A); an additional 40 microliters were assayed for alkaline phosphatase activity (B) using the procedures described in Methods. The avidin immune serum was prepared using the methods of Cho-Chung and Pitot (10). This immune serum yielded one sharp precipitin band when it was reacted with avidin on an ouchterlony plate.

Table I

Purification of Urea Carboxylase and Allophanate Hydrolase

Purification step	Total protein milligrams	Total activity, μmoles/min Urea Carboxylase	Total activity, μmoles/min Allophanate Hydrolase	Specific activity, μmoles/min/mg Urea Carboxylase	Specific activity, μmoles/min/mg Allophanate Hydrolase	A/U
1. Crude extract	5650	49.2	145	0.0087	0.026	2.94
2. Protamine	4800	29.0	127	0.0060	0.026	4.20
3. 35-45% ammonium sulfate fraction	180	15.9	82	0.088	0.45	5.13
4. DEAE-cellulose	41	13.6	55	0.336	1.36	4.05
5. Sucrose gradient tube #20	--	--	--	2.440	7.65	3.14

It is of interest to note that the urea carboxylase and allophanate hydrolase activities have also been found in a procaryotic organism, Chlorella vulgaris. In this organism, the two activities have been separated by chromatography on a brushite column (6). It has been found in several other fungal systems containing enzyme aggregates, e.g., the aromatic biosynthetic pathway in Neurospora crassa (8, 9) and the histidine biosynthetic pathway in Saccharomyces cerevisiae (5), that the activities associated with the aggregate in fungi are associated with separate proteins in bacteria (8, 9).

ACKNOWLEDGEMENT

We express our gratitude to Professor Boris Magasanik for his support and encouragement during the course of this work.

This work was supported by NIH grant 1-R01-AM-13894 to Boris Magasanik, and NIH grant R01-GM-19386, American Cancer Society grant PF-613 and Health Research and Services Foundation award 0-50 to T. G. Cooper. P. A. Whitney was supported by a Microbiology Training Grant 5-T1-GM-00602 to the Massachusetts Institute of Technology, Department of Biology.

[1] Present Address: Department of Genetics, University of Washington, Seattle, Washington 98195.

BIBLIOGRAPHY

1. Roon, R. J., and Levenberg, B., J. Biol. Chem. 243, 5213-5215 (1968).
2. Whitney, P.A., and Cooper, T. G., Biochem. Biophys. Res. Commun. 40, 814-819 (1970).
3. Whitney, P. A., and Cooper, T. G., J. Biol. Chem. 247, 1349-1353 (1972).
4. Case, M. E., and Giles, N. H., Proc. Natl. Acad. Sci. 68, 58-62 (1971).
5. Fink, G. R. , in Metabolic Regulation, (H. Vogel, ed.) 5th edition, 199-223, Academic Press, New York (1971).
6. Thompson, J. F., and Muenster, A-M. E., Biochem. Biophys. Res. Commun. 43, 1049-1055 (1971).
7. Knappe, J., Ann. Rev. Biochem. 39, 757-776 (1970).
8. Ahmed, S. I., and Giles, N. H., J. Bacteriol. 99, 231-237 (1969).
9. Berlyn, M. B., and Giles, N. H., J. Bacteriol. 99, 222-230 (1969).
10. Cho-Chung, Y. S., and Pitot, H. G., Eur. J. Biol. Chem. 3, 401-406 (1968).