INDICATION OF A SPECIFIC REGULATORY BINDING PROTEIN FOR ORNITHINETRANSCARBAMYLASE IN SACCHAROMYCES CEREVISIAE

J. BECHET AND J.M. WIAME

INSTITUT DE RECHERCHES, C.E.R.I.A., BRUXELLES 7 AND
LABORATOIRE DE MICROBIOLOGIE, UNIVERSITE LIBRE DE BRUXELLES

Received September 29, 1965

Ornithinetranscarbamylase (OTCase) catalyses one of the enzymatic steps in the biosynthesis of arginine.

In yeast, the pathway is similar to that found in Escherichia coli (Umbarger and Davis, 1962), except in the two following respects. First, the acetylation of glutamate occurs by recycling the acetyl from acetylornithine to glutamate (De Deken, 1963), thereby eliminating the necessity for acetylation of glutamate by acetyl CoA (Maas et al., 1953) and for deacetylation of acetylornithinase. Second, there are two distinct enzymes for the synthesis of carbamylphosphate and the regulation of one of them shows its specific integration in the arginine biosynthesis (Figure 1) (Lacroute et al., 1965). As in E. coli (Gorini and Maas, 1958), OTCase in yeast is strongly repressed by arginine (Bechet et al., 1962). The synthesis of the other enzymes as far as they have been tested is pleiotropically repressed (De Deken, 1963; Bechet et al., 1964).

In addition to the phenomenon of repression, arginine also inhibits (feedback inhibition) the reduction of acetylglutamate (De Deken, 1963) much as it inhibits the acetylation of glutamate in E. coli (Vyas and Maas, 1963). A mutation r arg_1^- leading to a genetically nonrepressible synthesis of OTCase has been obtained in S. cerevisiae (Bechet et al., 1964).

The purpose of this work is to indicate that the regulation of OTCase activity in yeast exhibits an additional manifestation, which involves the synthesis of a specific binding protein (SBP) for OTCase.

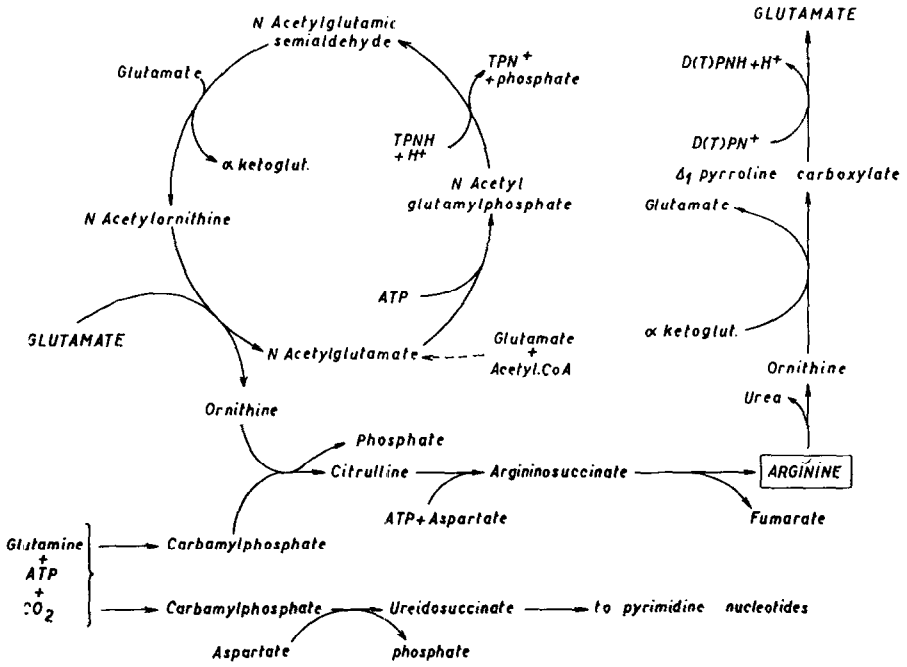


Figure 1. Biosynthesis and breakdown of arginine in Saccharomyces cerevisiae.

Materials and Methods. - Medium and conditions of growth : Cells were cultured in medium no. 140 with glucose 3 % and vitamins (Lacroute et al., 1965), aerated at 30°C on a gyratory shaker. Where indicated, L-arginine was added at 100 µg per ml.

Strains. - A detailed description of the history of the strains (mutations, crosses and genetical analysis) will be given in a separate paper. The strain Σ 1705d (r^+) is repressible and the strain Σ 1708c is a nonrepressible strain with the recessive mutation r arg_1^-.

OTCase Activity. - This was measured according to Jones, Spector and Lipmann (1955), with 0.05 M tris buffer pH 8, incubation at 37° for 30 min with either "permeabilized" cells or extracts. "Permeabilization" was performed at 30° for 30 min with nystatin (65 µg/ml) added to suspension of cells in water. The suspen-

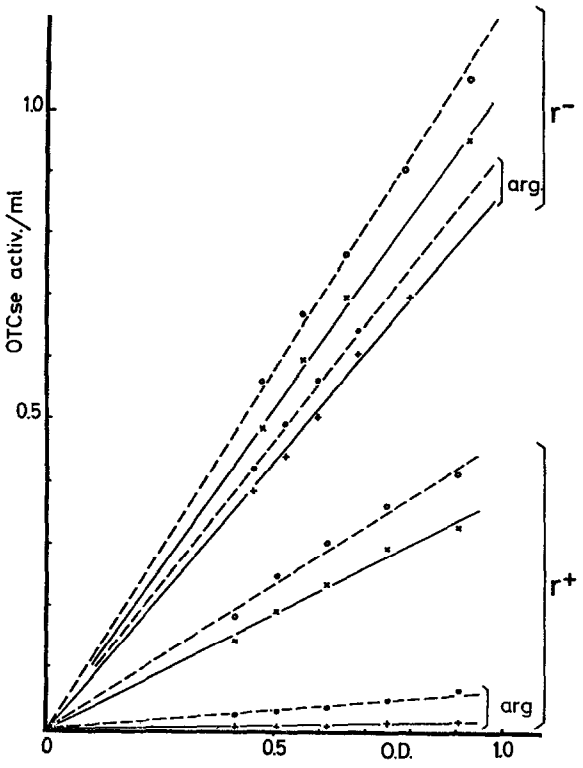


Figure 2. Differential rate of OTCase synthesis in repressible (r^+) and non repressible (r^-) strains. Cells are taken in exponential growth in minimal medium with or without arg. (100 µg/ml). OTCase activity, see Methods.

------ OTCase activity of permeabilized cells;
——— OTCase activity of extract obtained from the suspension of permeabilized cells.

sion contained between 0.3 to 0.6 mg/ml dry weight. The extracts were the same suspension of "permeabilized" cells, treated in the French pressure cell without eliminating the debris.

After incubation with ornithine and carbamylphosphate there was no need for the elimination of the cell material prior the determination of citrulline.

The Differential Rate of Synthesis : $p = \Delta$ Enzyme / Δ total cellular mass is a constant, characteristic for a strain and a given medium (Monod et al., 1952). Such values of p, for different media and r^+ (repressible) and r^- strains are shown in Figure 2. The OTCase activities of extracts obtained with the French pressure cell are compared with suspension of cells "permeabilized" by the antibiotic nystatin (Marini et al., 1961; Sutton et al., 1961). In few crucial determinations we have also "permeabilized" the cells with cetyltrimethylammonium-bromide (Davies, 1956); this gave the same result as nystatin. The "permeabilized" cells gave 80 to 90 % of the extracts activities, although for the r^+ strain grown

in the presence of arginine the differences between the two methods was more pronounced. Thus, all the activities were low, but the "permeabilized" cells exhibited only 20 % of the activity of the extracts.

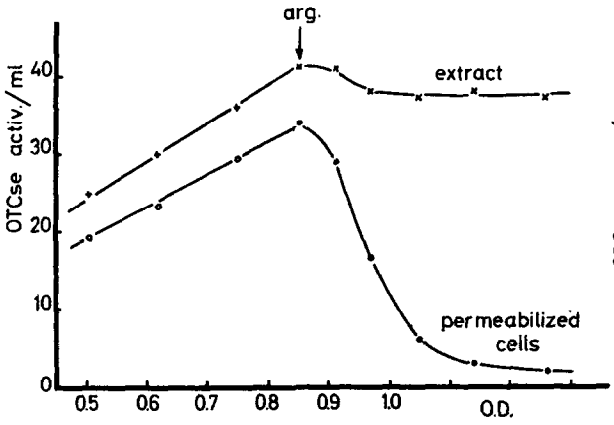
Chemicals. - The nystatin (Mycostatine) was kindly provided by the "Laboratoires Labaz" (Bruxelles); other chemicals are of commercial origin : actidione (L. Light and Co.), azetidine-carboxylic acid (Calbiochem), DL-ethionine and β-2-thienylalanine (Nutritional Biochemicals Corporation), DL-p-fluoro-phenylalanine (Hoffmann- La Roche).

Results. - OTCase activity of S. cerevisiae in transitory states : If the OTCase activity is followed during the shift from minimal medium to minimal plus arginine, strikingly different results are obtained between "permeabilized" and broken cells.

Figure 3 shows that the classical behavior is expressed with the broken cells (extract) : after addition of arginine the differential rate of enzyme synthesis is reduced, in agreement with the data reported in Figure 2. Contrasting with this, the activity of the "permeabilized" cells decreases rapidly and simulates a destruction of enzyme. The activity is reduced to 50 % after 0.10 generation (about 15 minutes). The decrease of activity ceases after 60 minutes ($\sim$ 0.5 doubling), at a value which is about 10 % of the initial value. This decrease in activity recalls observations which have been reported in a number of cases and named "deadaptation". Such examples have been believed to result from a disappearance of enzyme (Robertson and Halvorson, 1957).

However in the present case, as the activity is present in extract, such a hypothesis can be eliminated. At least in the phase which follows the addition of arginine, the phenomenon appears to be an "inactivation" of the enzyme.

The Role of Protein Synthesis in the Inactivation of OTCase. - Actidione (cycloheximide) is an antibiotic which inhibits protein synthesis (Kerridge, 1958; Ennin and Lubin, 1964; Siegel and Sisler, 1964). It stops lysine incorporation in less than 1 minute (at 2 µg/ml). As shown in Figure 4, actidione prevents also the decrease of OTCase activity measured in "permeabilized" cells.



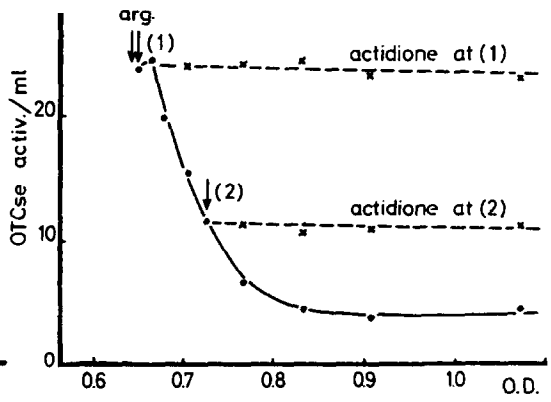


Figure 3. (Left) OTCase activity during shift from minimum to minimum + arginine with a repressible strain (Σ 1705d). Arginine is added at the times shown by the arrows.

Figure 4. (Right) Action of actidione on the inactivation of OTCase. Arginine added at the time shown by the arrow. The solid line indicates the evolution of the activity as shown in Figure 3. Actidione is added to two parts of this culture at (1) and at (2). The growth stops and the broken lines indicate the enzyme activity, which for the sake of comparison is ploted to the same ordinate as the sample of the growing culture taken at the same time.

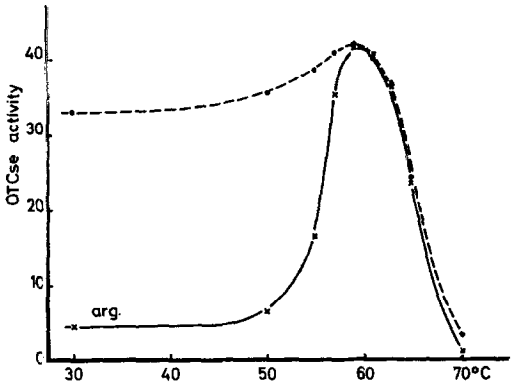
This indicates that the inactivation process involves the formation of a protein; the action of this protein is reversed when the cells are transformed in extract. This protein could act by a direct action on the OTCase or by a destruction of the substrates or product of the reaction used for the test of OTCase activity. It has been shown independently by Middelhoven (1964) and in this laboratory (*), that arginine induces in yeast the catabolic pathway through ornithine and Δ-1-pyrroline-carboxylic acid.

However, under the conditions of the assay no indication of a destruction

---

(*) The presence of an inducible Δ-1-pyrroline-carboxylicdehydrogenase active with DPN or TPN can be observed when the cell free extracts are fractionated with $(NH_4)_2SO_4$ to eliminate a very active constitutive pyrroline-carboxylic-reductase leading to proline. The dehydrogenase precipitates at 60 % $(NH_4)_2SO_4$ saturation, the reductase between 60 and 90 % (Messenguy, F., J. Bechet and J.M. Wiame, unpublished).

of carbamylphosphate, ornithine or citrulline has been obtained. Citrulline incubated with "permeabilized" cells is recovered and ornithine preincubated for 30 min in the absence of carbamylphosphate with "inactivated" cells is still entirely available for the citrulline synthesis with active cells. That carbamylphosphate remains available has been tested by showing that both active and inactive cells are equally able to perform the aspartic transcarbamylase activity even when the amount of carbamylphosphate has been reduced in the test from 5 µM to 1 µM (Yates and Pardee, 1957). Furthermore the results mentioned in the section on regulation mutants would be very difficult to explain on the basis of the hypothesis of an induced catabolic enzyme.



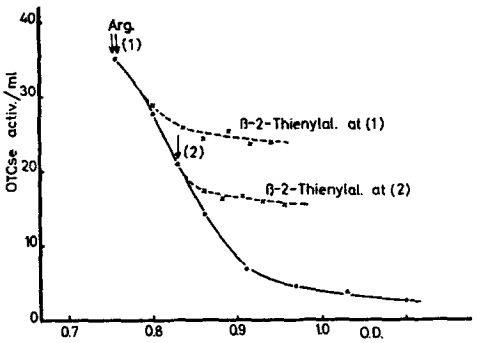


Figure 5. (Left) Thermoreactivation (5 min) of OTCase activity of "permeabilized" cells before (o) and after (x) addition of arginine for 1 hour. An exponentially growing culture is divided into two parts, one is cooled; to the other arginine is added for 1 hour as in the experiment described in Figure 3. The two types of suspensions are "permeabilized" and samples are heated for 5 min at different temperatures. After cooling the OTCase activity is measured.

x——x——, the arginine treated culture.
o——o——, the cells collected on minimal medium.

Figure 6. (Right) Action of ß-2-thienylalanine on the inactivation of OTCase activity in permeabilized cells. Conditions similar to Figure 4. Thienylalanine added at (1) and at (2).

We suggest that the inhibitory agent is a protein which acts directly on OTCase and that its operation is stoichiometric rather than catalytic.

Thermal Reactivation of the OTCase of "Permeabilized" Cells. - If samples of cells are taken 60 minutes after the addition of arginine, "permeabilized"

and then heated for 5 minutes at different temperatures, OTCase can be reactivated as shown in Figure 5. The recovery is complete after heating 5 minutes at 59°. Over 59°, heating results in a decrease of activity in the two types of cells. One may mention here that OTCase in "permeabilized" cells is more thermoresistant than in extract, where the activity disappears completely in 5 min at 59°.

Action of Amino-Acids Analogs. - The forementioned results suggested that amino-acids analogs could affect the inhibition of OTCase (Cohen et al., 1958). We have tried analogs at doses which inhibit growth.

β-2-Thienylalanine, an analog of phenylalanine (Munier and Cohen, 1959), reduces the inhibition of OTCase a short time after its addition (Figure 6). The three other, ethionine, para-fluoro-phenylalanine and azetidine-carboxylic acid have no or only a slight action.

Regulation Mutant. - The results of a similar experiment with a mutant which has lost its capacity for OTCase repression by arginine (strain Σ 1708c) are shown in Figure 7. The differential rate of synthesis of OTCase assayed in extracts is high and not significantly affected by growth in the presence of arginine (Figure 2). Furthermore, after addition of arginine there is only a slight change in the OTCase activity of permeabilized cells. This shows that the capacity to cancel OTCase activity disappears as a consequence of the mutation $r^+ \rightarrow r^-$. This mutation affects simultaneously the capacity to synthesize OTCase (tested in extracts) and the inhibition of the activity of this enzyme as assayed in permeabilized cells.

Discussion. - The data are the best explained by the occurence of a specific regulatory binding protein (SBP) for OTCase, under conditions of repression, with the result of a loss of in vivo activity after a very limited period of growth. The enzyme, estimated in extract or in heat reactivated "permeabilized" cells, does not disappear.

There are other cases of inhibitory proteins, specific for enzymes, such as the classical trypsin-inhibitor (Northrop et al., 1948; Laskowski and Laskowski,

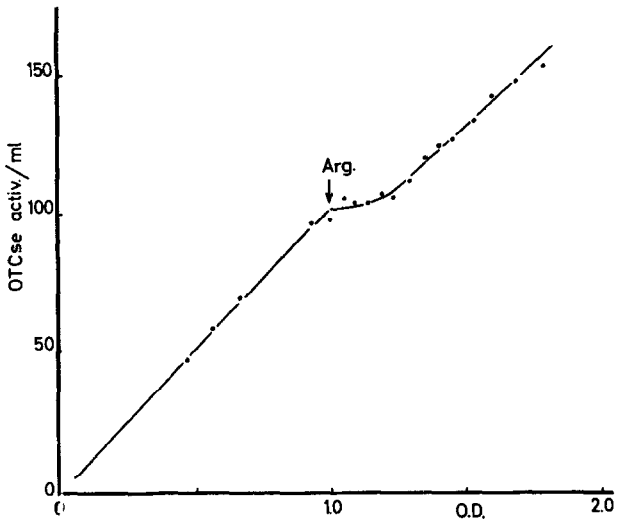


Figure 7. OTCase activity during shift from minimum to minimum + arginine with a non repressible strain (Σ 1708c). Arginine is added at the time shown by the arrow.

1954). An inhibitor of NAD pyrophosphatase, most probably protein in nature, has been found in different bacteria, and the fact that its amount changes with nutrition suggests a regulatory role (Schwartz et al., 1958). The evidence for SBP comes from experiments on heat lability and dissociation.

A systematic search in the cases of "deadaptation" or "cryptic" activities (Kaplan, 1965; Weinberg and Orton, 1965) might disclose similar phenomena.

The recent observation of Sussman (Sussman, 1965) on the disappearance of the activity of UDP-galactose polysaccharide transferase of Dictyostelium discoïdeum is particularly interesting since actidione stops this process.

A better insight on the meaning and mechanism of the regulation by a specific binding protein awaits new information, but the idea that such a mechanism of regulation would express a new type of genetical integration, in eucaryotic cells, is very stimulating.

Acknowledgements

The authors wish to thank Miss M. Demoulin for her devoted technical assistance. This work was supported by the Fonds de la Recherche Fondamentale Collective.

References

Bechet, J., J.M. Wiame, and M. Grenson, Arch. Intern. Physiol. Biochim., 70, 564 (1962).

Bechet, J., J.M. Wiame, and M. Grenson, Arch. Intern. Physiol. Biochim., 73, 137 (1964).

Cohen, G.N., H.O. Halvorson, and S. Spiegelman, in Microsomal particles and protein synthesis, ed. R.B. Roberts (Washington Academy of Sciences, 1958), p. 100.

Davies, A., J. Gen. Microbiol., 14, 425 (1956).

De Deken, R.H., Biochim. Biophys. Acta, 78, 606 (1963).

De Deken, R.H., Biochem. Biophys. Res. Comm., 8, 462 (1962).

Ennis, H.L., and M. Lubin, Science, 146, 1475 (1964).

Gorini, L., and W.K. Maas, in Chemical Basis of Development, ed. W.D. McElroy and B. Glass (Baltimore : Johns Hopkins Press, 1958).

Jones, M.E., L. Spector, and F. Lipmann, J. Amer. Chem. Soc., 77, 819 (1955).

Kaplan, J.G., J. Gen. Physiol., 48, 873 (1965).

Kerridge, D., J. Gen. Microbiol., 19, 497 (1958).

Lacroute, F., A. Piérard, M. Grenson, and J.M. Wiame, J. Gen. Microbiol., 40, 127 (1965).

Laskowski, M., and M. Laskowski Jr., Adv. Prot. Chem., 9, 203 (1954).

Maas, W.K., C.D. Novelli, and F. Lipmann, Proc. Nat. Acad. Sc. Washington, 39, 1004 (1953).

Marini, F., P.M. Arnow, and J.O. Lampen, J. Gen. Microbiol., 24, 51 (1961).

Middelhoven, W.J., Biochim. Biophys. Acta, 93, 650 (1964).

Monod, J., A.M. Pappenheimer, and Cohen-Bazire, G., Biochim. Biophys. Acta, 9, 648 (1952).

Munier, R., and G.N. Cohen, Biochim. Biophys. Acta, 31, 378 (1959).

Northrop, J.H., M. Kunitz, and R.M. Herriott, in Cristalline Enzymes (New York : Columbia University Press, 1948).

Robertson, J.J., and H.O. Halvorson, J. Bacteriol., 73, 186 (1957).

Schwartz, M.D., N.O. Kaplan, and M.F. Lamborg, J. Biol. Chem., 232, 1051 (1958).

Siegel, M.R., and H.D. Sisler, Biochim. Biophys. Acta, 87, 70 (1964).

Sussman, M., Biochem. Biophys. Res. Comm., 18, 763 (1965).

Sutton, D.D., P.M. Arnow, and J.O. Lampen, Proc. Soc. Exptl. Biol. Med., 108, 170 (1961).

Umbarger, E., and B.D. Davis, in The Bacteria, ed. I. Gunsalus and R. Stanier (New York : Academic Press, 1962), vol. III.

Vyas, S., and W.K. Maas, Arch. Biochem. Biophys., 100, 542 (1963).

Weinberg, R., and W.L. Orton, J. Bacteriol., 89, 740 (1965).

Yates, R.A., and A.B. Pardee, J. Biol. Chem., 227, 677 (1957). (Except that pH is 8.0).