REGULATION OF ARGINASE LEVELS BY UREA AND INTERMEDIATES OF THE KREBS-HENSELEIT CYCLE IN *SACCHAROMYCES CEREVISIAE*

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1. Introduction

The importance of Krebs-Henseleit cycle amino acids and urea as nitrogen sources in plants and microorganisms is now well documented [1–4]. Furthermore, it is clear that arginine, being particularly rich in nitrogen, can be recycled to form other nitrogenous compounds [5]. The importance of urea in such metabolic recycling of nitrogen is indicated by the ability of certain microorganisms and higher plants to utilize this compound as a sole source of nitrogen [6–8]. In Saccharomyces, arginine is known to regulate its biosynthesis and metabolism by (a) feedback inhibition of N-γ-acetylglutamate reductase [9]; (b) enzyme repression within the biosynthetic pathway [10]; (c) inhibition of ornithine transcarbamylase [11]; and (d) induction of arginase [12]. However, apart from the work of Middelhoven [12, 13], little has been published on the regulation of arginase in this organism by products or intermediates of the Krebs-Henseleit cycle. Considering the apparent metabolic importance of urea in microorganisms, it follows that the accumulation of this compound may affect the cycling of nitrogen and operation of the Krebs-Henseleit cycle. In this respect, it is of interest to note that urea is readily hydrolyzed to ammonia [14], and this product inhibits the synthesis of arginase in Saccharomyces [15].

The present studies were conducted to examine the possibility that urea may regulate the operation of this cycle by controlling the activity of arginase. The levels of this enzyme were substantially altered by varying the nitrogenous components of the culture medium. It is concluded, from the data obtained, that urea regulates the level of arginase by enzyme repression.

2. Materials and methods

2.1. *Culture conditions*

Saccharomyces cerevisiae (ATCC 9763) was grown in a complete defined medium (basic culture medium), totally devoid of any nitrogenous substrates or in the basic culture medium supplemented with the various nitrogenous compounds. The basic culture medium contained (per 100 ml): 50 μg thiamine, 5 mg inositol, 500 μg each of calcium pantothenate and niacin, 2 μg biotin, 85 mg KCl, 1.5 mg $MgSO_4 \cdot 2H_2O$, 25 mg $CaCl_2 \cdot H_2O$, 110 mg KH_2PO_4, 0.5 mg each of $MnSO_4 \cdot H_2O$ and $FeCl_3 \cdot 6H_2O$, 1 g potassium citrate, 1.2 g citric acid and 10 g glucose.

In all work, *S. cerevisiae* was subcultured aerobically at 30° for 24 hr in the basic culture medium supplemented with 4 mg each of DL-leucine, DL-valine, L-cysteine, DL-phenylalanine, DL-threonine, DL-alanine, L-aspartic acid, L-lysine HCl and DL-serine, 5 mg L-glutamic acid, 2 mg each of L-isoleucine, L-tryptophan, L-tyrosine, L-histidine $HCl \cdot H_2O$ and glycine, and 1 mg L-proline. Cells from this culture were used to inoculate basic culture medium with or without the addition of nitrogenous compounds. Cells were then grown for the desired length of time at 30° with aeration.

2.2. *Preparation of cell-free extracts*

After centrifugation, the cells were washed 3 times with cold sterilized distilled water and suspended in 10 mM 2-mercaptoethanol containing 1% $NaHCO_3$, 0.5 ml being used per g f. wt. of cells. The cells were then disintegrated by treatment in a Fisher Ultrasonic generator at 2°. The suspension was then diluted 4-fold by addition of 0.01 M potassium phosphate

(pH 7.5), and centrifuged for 10 min at 18,000 *g*. The supernatant was stored at 4°. No loss of arginase activity was observed when such extracts were stored at 2–4° for periods up to 5 days, but losses of 50–60% occurred after storage periods of 10 days.

2.3. *Sephadex G-25 treatment*

In experiments involving cycloheximide treatment, the cell-free extracts were partially purified using Sephadex G-25. All procedures were carried out at 4°. The cell-free extract (3 ml), containing approximately 25–35 mg protein, was applied to a 1.6 × 20 cm column of Sephadex G-25 and eluted with 0.01 M potassium phosphate (pH 7.5). Fractions of 3 ml were collected at a flow rate of 60 ml/hr. The bulk of the applied protein was collected in fractions 6–8. These fractions, when pooled and assayed for enzyme activity, showed that approximately 80–90% of the initial arginase activity was present. A 6–8 fold increase in specific enzyme activity was routinely obtained by this gel filtration.

2.4. *Assay of arginase activity*

Enzyme activation was achieved by pre-incubation with 10 μmoles manganese maleate (pH 7.0) for 30 min at 30°. Arginase activity was then assayed by incubation of the activated enzyme with 140 μmoles of L-arginine (free base), pH 9.2, in a final volume of 0.5 ml. After incubation for 10 min at 30°, 4 ml of Hycel urea nitrogen reagent were added and color development was achieved in a boiling water bath for 12 min followed by immediate chilling of the tubes for 3 min in an ice bath. Absorbance was measured at 490 nm. Control systems, without enzyme or substrate, were included. The quantity of urea formed was determined by reference to a standard curve prepared with a standard urea solution. Protein content was determined colorimetrically [16].

3. Results

3.1. *Effects of various nitrogenous compounds on levels of arginase during growth*

In order to determine changes in the levels of arginase present in crude extracts, cells were harvested at the end of growth periods on various supplemented media as shown in table 1. It is clear that the specific activity of arginase differed in cells grown in the presence of different nitrogenous compounds. Specific enzyme activities were highest in the presence of L-arginine, (2×10^{-2} M) and lowest in the presence

Table 1
The influence of arginine, ornithine, urea and $(NH_4)_2SO_4$ on the specific activity of arginase.

Basic culture medium supplement	Concentration (M)	Specific activity (μmoles urea/hr/mg protein)
None		11.30
L-arginine	2×10^{-2}	38.5
L-arginine	2×10^{-1}	9.60
Urea	2×10^{-2}	4.0
Urea	2×10^{-1}	3.00
L-ornithine	2×10^{-2}	8.00
$(NH_4)_2SO_4$	2×10^{-2}	4.20
L-arginine-HCl + urea	2×10^{-2} 2×10^{-2}	5.10
L-arginine-HCl + L-ornithine	2×10^{-2} 2×10^{-2}	23.4
L-arginine-HCl + $(NH_4)_2SO_4$	2×10^{-2} 2×10^{-2}	7.20

Table 2
Derepression of arginase by L-arginine (2×10^{-2} M) as a function of growth time.

Culture period (hr)	Specific activity of arginase (μmoles urea/hr/mg protein)
0	1.2
0.5	6.6
1	8.0
1.5	8.5
2	9.6
2.5	10.6
3	10.8
3.5	11.0
4	13.2
5	15.0
6	16.8

The yeast cells were initially cultured in the basic culture medium supplemented with 2×10^{-2} M urea for 4 hr before transfer to the arginine-supplemented medium.

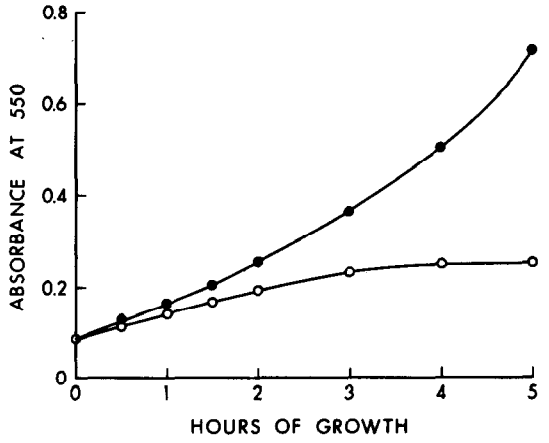

Fig. 1. The effect of cycloheximide on growth. Cells were initially cultured (for 4 hr at 30°) in the basic medium supplemented with 2×10^{-2} M urea. After harvesting, aliquots of the cells were used to inoculate fresh basic culture media supplemented with 2×10^{-2} M urea, in the presence (○——○) and absence (●——●) of cycloheximide. Data are the average for 3 separate experiments.

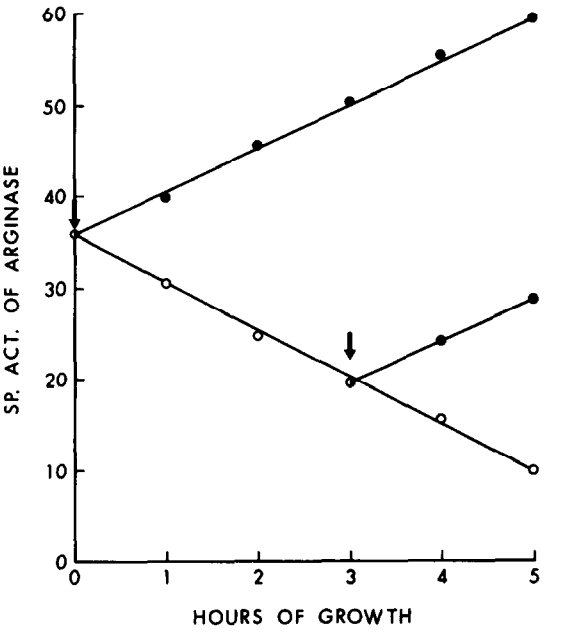

Fig. 2. The effect of cycloheximide on the activity of arginase. Cells were cultured as in fig. 1. Arginase activities were assayed for cultures grown in the presence (●) and absence (○) of cycloheximide after Sephadex G-25 treatment. The arrows indicate additions of cycloheximide (10 μg/ml of medium). Data are the average of 4 separate experiments.

of urea and $(NH_4)_2SO_4$, (2×10^{-2} M). Cells cultured without a nitrogen supplement had a specific enzyme activity between these extremes. When the arginine concentration was increased from 2×10^{-2} M to 2×10^{-1} M, the specific activity of arginase decreased from 36.0 to 9.60. This may be associated with accumulation of urea formed from arginine as the addition of urea to the basic medium resulted in decreases in specific enzyme activity (table 1). Various amounts of arginine added with ornithine, urea or $(NH_4)_2SO_4$, tended to increase the levels of arginase to some extent. Ornithine alone, when present in the culture media, decreased enzyme activity below that of the control. Although not shown in table 1, these supplements to the basic culture medium also altered the rate of growth. For example, after 5 hr of growth, under these conditions from a standard inoculum, the amount of dry cells harvested were 96, 124 and 187 mg for the basic, arginine-supplemented and urea-supplemented cultures, respectively. The large amount of growth in the presence of urea clearly suggests that this compound can be readily utilized as a nitrogen source.

From the data in table 1 it appears that the products of arginase activity repress or inhibit the activity of this enzyme while the substrate elevates the levels of arginase when added at low concentration. In later experiments, the derepression of arginase by arginine was followed as a function of time (table 2). Within 30 min of transfer from a urea-containing medium, the specific activity of arginase rose more than 5 times, and after 6 hr of growth was 14 times greater than that of the urea-grown cells.

3.2. *The effect of cycloheximide on the repression of arginase by urea*

In these experiments, cycloheximide, at a concentration of 10 μg/ml, was added to samples of culture media which had been supplemented with urea. For such experiments, the cells were initially harvested after 4 hr incubation in the basic medium supplemented with urea (2×10^{-2} M). Growth was measured spectrophotometrically. It is clear from fig. 1 that cycloheximide inhibited growth of the cells. When accompanying changes in arginase were examined (fig. 2), it was clear that culture in the presence of urea resulted in low levels of arginase which decreased further as

growth under these conditions continued. These decreases were not, however, observed in the presence of cycloheximide. Under these conditions, the specific activity of arginase rose, the rise occurring immediately after addition of cycloheximide. However, these increases were more gradual and of smaller magnitude when compared with the increases caused by L-arginine, in the earlier experiments.

4. Discussion

The finding that arginase levels are altered by supplementing the medium with various nitrogenous compounds (table 1) suggests that the activity of this enzyme in Saccharomyces is closely regulated. In agreement with the present studies, Middelhoven [12] concluded that synthesis of arginase was induced by its substrate. In contrast, addition of ornithine and urea to the basic culture medium (table 1) resulted in substantially lower levels of arginase. In more detailed experiments (Chan and Cossins, to be published) it was clear that this effect of ornithine could be readily removed by Sephadex G-25 treatment, suggesting that ornithine, present in the crude cell-free extracts, inhibited arginase activity to some extent. This was, in fact, confirmed in kinetic studies which clearly showed that arginase was competitively inhibited by ornithine *in vitro*. In contrast, the decrease of arginase levels associated with culture in the presence of urea could not be removed by gel filtration and urea did not affect arginase activity *in vitro*. These findings, therefore, suggest that urea may be effective in repressing synthesis of this enzyme. Support for this contention was obtained in the experiment summarized in table 2, which showed that cells, cultured initially in the presence of urea, rapidly synthesized high levels of arginase on transfer to an arginine supplemented medium. A similar derepression of this enzyme was found to occur when cells, maintained in the presence of $(NH_4)_2SO_4$, were transferred to a minimal medium [15].

It is generally agreed that cycloheximide is useful in the study of control mechanisms at the gene level [17]. In this respect, synthesis of the repressor, which is known to be a polymeric protein [18, 19], will be inhibited by this antibiotic. Cycloheximide, by inhibiting protein synthesis, will, therefore, decrease formation of both the repressor and arginase. This inhibitory effect will, however, be a differential one as only limited repressor molecules will be synthesized per genome in each generation [20, 21]. It is logical to assume, therefore, that synthesis of the repressor will be terminated well before the synthesis of arginase stops. At the same time, the synthesis of other proteins will stop gradually resulting in an apparent increase in the specific activity of arginase. This situation would also apply if cycloheximide were added at intervals to the cells during growth.

In experiments employing cycloheximide (fig. 1 and 2), it was observed that growth was slowly and progressively inhibited with time. However, following addition of the antibiotic to urea-supplemented cultures the specific activity of arginase began to increase within 30 min. Such increases in enzyme activity were also slow and progressive. This effect suggests that urea may serve as a specific metabolite capable of activating the repressor for arginase. On the other hand, increased synthesis of this enzyme by arginine would be associated with inactivation of the repressor.

In conclusion, it appears that both products of arginase activity can regulate this reaction of the Krebs-Henseleit cycle. In the case of ornithine, control appears to be exerted by product inhibition whereas enzyme repression occurs in the presence of urea. As urea appears to be an important source of nitrogen in Saccharomyces, repression of this key enzyme in the Krebs-Henseleit cycle will have considerable physiological significance. More detailed studies confirming this regulatory role of urea will be the subject of a further publication.

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