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Effect of Hydroxylysine on the Biosynthesis of Lysine in *Saccharomyces*

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Hydroxylysine acts as a growth inhibitor of *Saccharomyces* for a certain period of time. The inhibition is concentration-dependent and is reversed by a small amount of lysine in the medium. After the growth-inhibitory period, the wild-type cells are able to grow rapidly even in the presence of hydroxylysine. Both lysine auxotrophs and wild-type cells are unable to utilize hydroxylysine in place of lysine. Hydroxylysine, mimicking lysine, controls the biosynthesis of lysine and thereby limits the availability of biosynthetic lysine to the cells. Hydroxylysine affects the biosynthesis of lysine at a number of enzymatic steps. Accumulation of homocitric acid, the first intermediate of lysine biosynthesis, in the mutant strains 19B and AB9 is reduced significantly in the presence of hydroxylysine. Hydroxylysine, like lysine, exerts a significant inhibition in vitro on the homocitric acid-synthesizing activity. Enzymes following the α-aminoadipic acid step respond in a noncoordinate fashion to hydroxylysine. Level of the enzyme saccharopine reductase, but not of α-aminoadipic acid reductase or saccharopine dehydrogenase, is reduced significantly. These regulatory effects of hydroxylysine are similar to those observed for lysine.

Hydroxylysine is known to control the biosynthesis of lysine in bacteria by feedback inhibition and repression in a manner similar to the regulation caused by lysine itself. Hydroxylysine inhibits the growth of the organism by limiting the cellular source of lysine (6). Whereas the biosynthesis of lysine in bacteria occurs via the diaminopimelic acid pathway (20, 22; C. Gilvarg, Fed. Proc. 19:948-952, 1960), the biosynthesis of this amino acid in *Saccharomyces* and other fungi occurs through a completely different pathway known as the homocitric acid pathway (5, 12, 16-19; Fig. 1). Results presented in this report describe the effect of hydroxylysine on the growth of the organism and the biosynthesis of lysine in *Saccharomyces*.

MATERIALS AND METHODS

Organisms and growth conditions. Unless otherwise stated, wild-type *Saccharomyces* (WL-1) was grown in liquid synthetic medium (Difco Yeast Nitrogen Base) without any amino acid. Lysine auxotrophic strains 19B (lys_4), AB9 (lys_5), 46964 (lys_{13}) and 74615 (lys_{14}) were grown in the same medium, supplemented with L-lysine as desired. Cultures were grown at 30 C with the use of a water bath shaker. Cell densities were determined by measuring the optical density at 550 nm with a Coleman spectrophotometer.

Isolation of radioactive products from culture supernatant fluid. Mutant strain 19B was grown in a 250-ml Erlenmeyer flask containing 50 ml of liquid synthetic medium supplemented with [*1*-^{14}C]sodium acetate (25 μCi) and lysine (3.5 μmoles) with or without hydroxylysine (70 μmoles). After 96 hr, cells were harvested by centrifugation and dry weight was determined. Culture supernatant fluid from each flask was lyophilized, dissolved in a small amount (1 to 2 ml) of distilled water, neutralized with dilute NaOH solution, and chromatographed on a column (1 by 30 cm) of Dowex-1-formate (3, 11). Fractions of 5 ml were collected, and a 1-ml sample from each fraction was dried and counted in a Beckman liquid scintillation spectrometer with a toluene-ethanol solvent system. Fractions containing homocitric, homoaconitic, and α-ketoglutaric acids were identified (4, 11), and radioactivity in the respective products was expressed as dry weight of cell mass per milligram.

Enzyme assay. The activity of the homocitric acid synthetase and the enzymes involved in the conversion of α-aminoadipic acid to lysine was measured in the cell-free preparations of 40-hr-old cultures grown in different concentrations of lysine or lysine and hydroxylysine. Cell extract was prepared by the method described previously (15).

Homocitric acid synthetase activity was determined by the procedure described by Strassman and Ceci (16). Formation of radioactive homocitric acid from [*1*-^{14}C] sodium acetate was ascertained by column chromatography.

α-Aminoadipic acid reductase (EC 1.2.1.26) activity responsible for the conversion of α-aminoadipic acid to α-aminoadipic acid-δ-semialdehyde in the cell-free

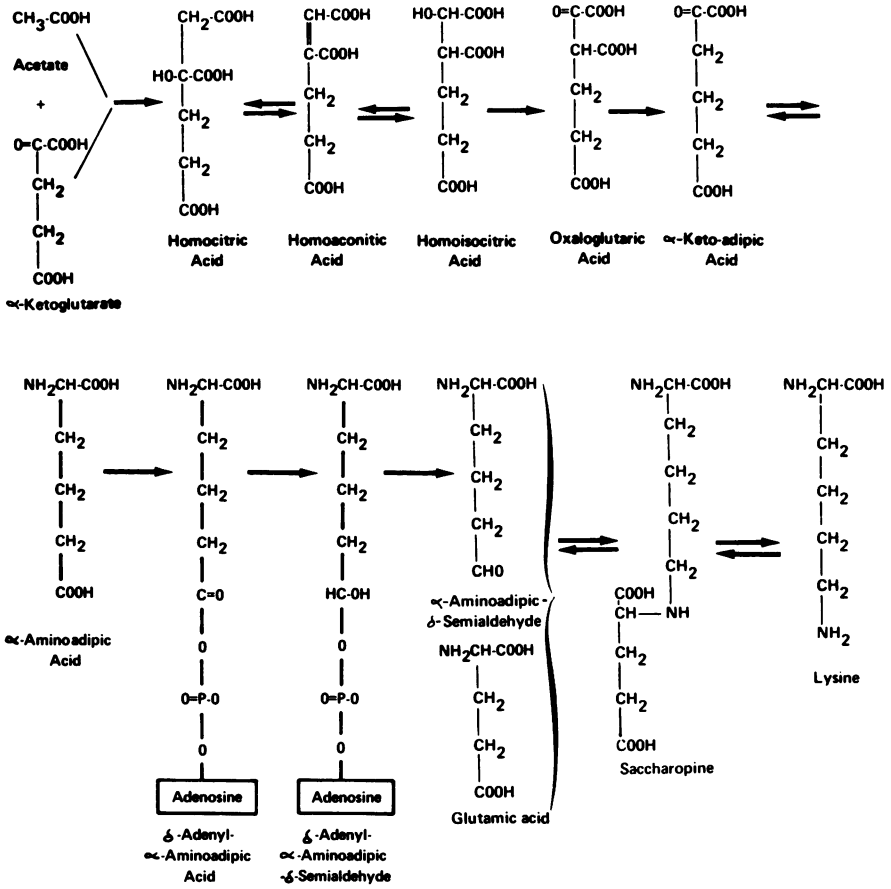

FIG. 1. *Proposed homocitric acid pathway for the biosynthesis of lysine.*

preparations of WL-1 was measured by the procedure described by Sagisaka and Shimura (13). A typical reaction mixture contained: α-aminoadipic acid, 2.5 μmoles; adenosine triphosphate, 3.0 μmoles; $MgCl_2$, 2.0 μmoles; reduced glutathione, 80 μg; reduced nicotinamide adenine dinucleotide phosphate (NADPH), 0.25 mg; tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 8.0), 200 μmoles; and appropriate amounts of cell-free preparations equivalent to 4 to 5 mg of protein in a total volume of 0.8 ml. The reaction mixture was incubated for 3 hr at 30 C, and the formation of α-aminoadipic acid-δ-semialdehyde was measured by the addition of (*p*)-dimethylaminobenzaldehyde into the reaction mixture. The result was calculated in terms of absorbancy at 460 nm per mg of protein.

Saccharopine reductase (EC 1.5.1.9) activity responsible for the conversion of α-aminoadipic acid-δ-semialdehyde to saccharopine was measured by determining the formation of α-aminoadipic acid-δ-semialdehyde from saccharopine by the procedure described by Jones and Broquist (9). The reaction mixture contained: saccharopine, 2.0 μmoles; nicotinamide adenine dinucleotide phosphate (NADP), 2 μmoles; glycine-NaOH buffer (*p*H 9.5), 100 μmoles; and cell-free preparations equivalent to 4 to 6 mg of protein in a total volume of 1.0 ml. The reaction mixture was incubated at 30 C for 3 hr. Formation of α-aminoadipic acid-δ-semialdehyde was determined by the procedure described for aminoadipic acid reductase and expressed in terms of absorbancy at 460 nm per mg of protein.

Saccharopine dehydrogenase (EC 1.5.1.7) activity responsible for the conversion of saccharopine to lysine was assayed by the procedure described by Saunders and Broquist (14) by following the reaction in reverse direction. Reaction mixture contained: L-lysine, 1.5 μmoles; α-ketoglutarate, 1.5 μmoles; nicotinamide adenine dinucleotide, reduced (NADH), 0.56 μmole; phosphate buffer (*p*H 7.0), 150 μmoles; and cell-free preparations equivalent to 4 to 5 mg of protein in a total volume of 1.5 ml. The reaction was carried out at room temperature, and the activity was expressed in terms of absorbancy at 340 nm per mg of protein with an Hitachi Perkin-Elmer spectrophotometer.

Chemicals. δ-Hydroxylysine, *N*-formyllysine, NADPH, NADH, and NADP were obtained from Sigma Chemical Co., St. Louis, Mo. The [*l*-^{14}C]sodium acetate was bought from International Chemical and Nuclear Corp. Saccharopine was the generous gift of S. Darling and P. O. Larsen.

RESULTS

Effect of hydroxylysine on growth of wild-type and lysine auxotrophs of Saccharomyces. Synthetic media supplemented with hydroxylysine failed to support the growth of the lysine auxotrophic strain 19B (Fig. 2). Similar results were obtained with strains AB9, 46964, and 74615. Wild-type *Saccharomyces* (WL-1) grew well in the synthetic minimal medium (Fig. 3A), and the growth was inhibited for a certain period of time in the presence of hydroxylysine. The inhibition was proportional to the concentration of hydroxylysine, ranging from 0.05 mM to 1.2 mM. No growth was observed for WL-1 up to 60 hr in the presence of 1.2 mM hydroxylysine (Fig. 3B). However, on further incubation, a rapid growth was observed. When cells from the delayed log phase were transferred into fresh medium containing 1.2 mM hydroxylysine, a normal growth (resistant phenotype) was observed. The inhibitory effect of hydroxylysine was overcome by supplementing the medium with lysine. It was found that the presence of 0.07 mM lysine in the growth medium of WL-1 was sufficient to overcome the inhibitory effect of 1.2 mM hydroxylysine. Similar results were obtained with the mutants. Unlike hydroxylysine, *N*-formyllysine failed to inhibit the growth of WL-1.

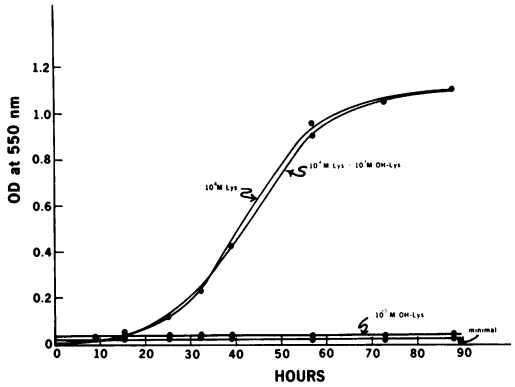

FIG. 2. *Growth response of mutant 19B (lys_4). Mutant was inoculated in the minimal medium, minimal medium supplemented with hydroxylysine (OH-Lys) alone, lysine alone, and lysine and hydroxylysine.*

Because hydroxylysine inhibited the growth of *Saccharomyces*, and because a small amount of lysine reversed the inhibitory effect of hydroxylysine, the effect of hydroxylysine on the biosynthesis of lysine was considered. For this purpose, mutant 19B was grown in the presence of *l*-^{14}C-sodium acetate and 3.5 μmoles of lysine and, in a separate experiment, in the presence of *l*-^{14}C-sodium acetate, 3.5 μmoles of lysine, and 70 μmoles of hydroxylysine. Radioactivity of accumulated homocitric, homoaconitic, and α-ketoglutaric acids (4, 11) was compared in the presence and in the absence of hydroxylysine (Table 1). In the presence of hydroxylysine, a significant reduction of radioactivity was observed for homocitric acid; a small reduction was observed for homoaconitic acid; and no reduction was observed for the α-ketoglutaric acid. A similar effect on the accumulation of homocitric acid was also observed with AB9 strain.

Effect of hydroxylysine on biosynthesis of enzymes involved in conversion of α-aminoadipic acid to lysine. Effect of hydroxylysine in vivo on α-aminoadipic acid reductase, saccharopine reductase, and saccharopine dehydrogenase was determined by growing WL-1 and three mutant strains (AB9, 46964, and 74615) in the presence of 3.5 μmoles of lysine and different amounts of

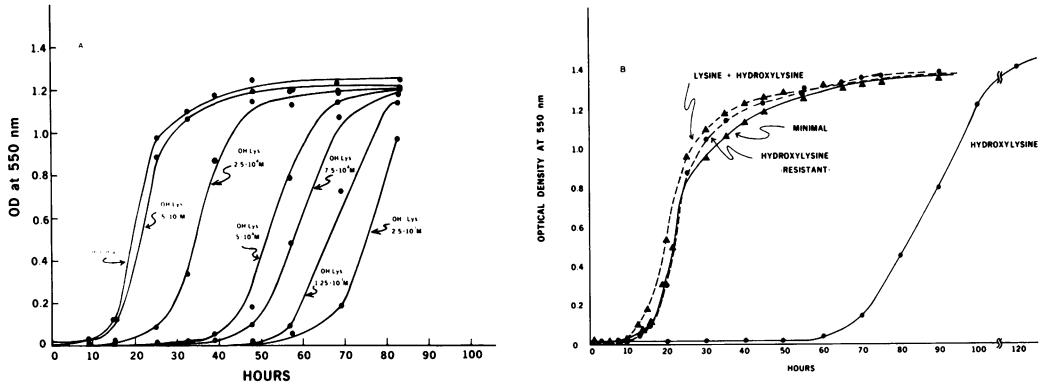

FIG. 3. *A, Growth response of wild-type Saccharomyces (WL-1). Cells were inoculated in the minimal medium and in minimal medium supplemented with increasing concentrations of hydroxylysine (OH-Lys). B, Effect of hydroxylysine on the growth of WL-1. Symbols: (●——●) minimal medium supplemented with 1.2 mM hydroxylysine, (●- -●) late log-phase cells (●——●) transferred to fresh medium containing 1.2 mM hydroxylysine, (▲—▲) minimal medium, and (▲---▲) minimal medium supplemented with 0.07 mM lysine and 1.2 mM hydroxylysine.*

hydroxylysine in the media. The results were compared with those of lysine by growing these organisms in the presence of different amounts of lysine only. Of the enzymes mentioned, the level of saccharopine reductase was reduced significantly by the presence of hydroxylysine in the medium, and the effect was similar to that of lysine (Fig. 4). No significant effect of lysine or hydroxylysine was observed on the level of α-aminoadipic acid reductase of WL-1. The level of saccharopine dehydrogenase in WL-1 was stimulated to some extent by lysine, although hydroxylysine was relatively ineffective (Fig. 4). The possibility was considered that an intracellular pool of lysine in the wild strain may have been responsible for the lack of reduction of α-aminoadipic acid reductase and saccharopine dehydrogenase levels. Strain AB9 lacks α-aminoadipic acid reductase activity (15), and strains 46964 and 74615 lack saccharopine reductase activity (Sinha and Bhattacharjee, *unpublished data*). The saccharopine reductase activity of AB9 and the α-aminoadipic acid reductase, as well as the saccharopine dehydrogenase activities of 46964 and 74615, exhibited similar effects from lysine and hydroxylysine as that described for WL-1.

Effect of hydroxylysine in vitro on homocitric acid synthetase, α-aminoadipic acid reductase, saccharopine reductase, and saccharopine dehydrogenase. It has been shown earlier that the addition of hydroxylysine to the growth medium of strain 19B resulted in a significant reduction of the accumulation of homocitric acid in the medium (Table 1). As the homocitric acid synthetase activity of *Saccharomyces* has been reported to be inhibited in vitro by lysine (10, 21), it was thought that the observed effect of hydroxylysine on the accumulation of homocitric acid might be attributable to the same mechanism. When the homocitric acid synthetase activity of the cell-free extract of WL-1 was measured in the presence of either lysine or hydroxylysine and compared with that in the absence of the amino acids in the system, it was found that hydroxylysine, like lysine, was a potent inhibitor of the homocitric acid synthetase activity in *Saccharomyces* (Table 2). In contrast to the in vitro effect of hydroxylysine or lysine on the activity of homocitric acid synthetase, addition of either of these amino acids to the reaction mixtures of α-aminoadipic acid reductase, saccharopine reductase, and saccharopine dehydrogenase produced no detectable effect.

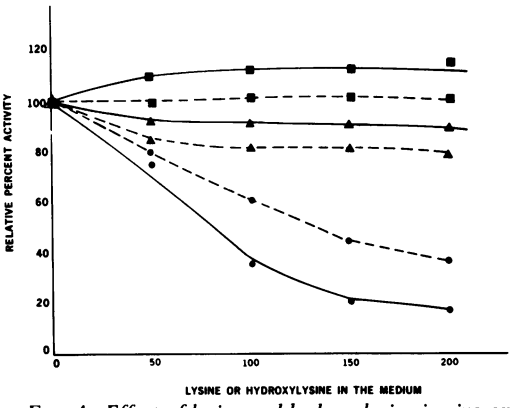

FIG. 4. *Effect of lysine and hydroxylysine in vivo on the level of α-aminoadipic acid reductase, saccharopine reductase, and saccharopine dehydrogenase. WL-1 was grown in the minimal medium as well as in the increasing concentrations (expressed in micromoles) of lysine (——) or 3.5 μmoles of lysine and increasing concentrations of hydroxylysine (- - -). Activity was determined in the individual culture extract for α-aminoadipic acid reductase (▲), saccharopine reductase (●), and saccharopine dehydrogenase (■).*

TABLE 1. *Effect of hydroxylysine on the accumulation of homocitric, homoaconitic, and α-ketoglutaric acids in the mutant ly_4*[a]

Growth conditions for ly_4	Homocitric acid (counts/min)	Homoaconitic acid (counts/min)	α-Ketoglutaric acid (counts/min)	Dry wt of cell mass
Lysine (3.5 μmoles)	45,129	8,337	33,037	34
Lysine (3.5 μmoles), hydroxylysine (70 μmoles)	25,052	6,651	34,975	30

[a] Mutant accumulated products were separated by column chromatography, and fractions for each product were pooled. The radioactivity in each of the products is expressed in terms of dry weight of cell mass per milligram.

TABLE 2. *Effect of lysine and hydroxylysine in vitro on enzymatic formation of homocitric acid*

Addition to reaction mixture[a]	Homocitric acid fraction (counts per min per ml)	Inhibition (%)
None	20,650	0
Lysine (50 μmoles)	4,272	79
Hydroxylysine (50 μmoles)	5,578	73

[a] Reaction mixture contained: sodium acetate (with 80 μCi of acetate-*1*-^{14}C), 50 μmoles; α-ketoglutarate, 50 μmoles; $MgCl_2$, 15 μmoles; adenosine triphosphate, 15 μmoles; CoA, 1 μmole; phosphate buffer (*p*H 7.2), 100 μmoles; dialyzed cell-free preparations of WL-1 equivalent to 6 mg of protein in a total volume of 2.5 ml. Incubation was carried out under nitrogen atmosphere at 30 C for 90 min.

DISCUSSION

As in the case of bacteria (6), hydroxylysine seems to inhibit the growth of *Saccharomyces* by affecting the biosynthesis of lysine. Lysine auxotrophs fail to substitute hydroxylysine for lysine as the growth requirement. However, a small amount of lysine in the medium is sufficient to support the growth of the mutant even in the presence of large amounts of hydroxylysine. Similarly, in the case of the wild-type organism, the inhibitory effect of hydroxylysine is reversed by a small amount of lysine in the medium. Prolonged exposure to a high level of hydroxylysine alone apparently makes the organism insensitive to hydroxylysine. However, the effect is phenotypic, and the genetic and biochemical mechanisms of this insensitivity remain unknown.

Formation of homocitric acid, the first intermediate of the lysine pathway, is known to be under end-product control in both *Saccharomyces* (10, 21) and *Neurospora* (8). In addition, the accumulation of homocitric, homoaconitic, and homoisocitric acids in appropriate mutants of *Saccharomyces* (1, 2) and *Rhodotorula* (7) is known to be sensitive to lysine. Accumulation of homocitric acid in vivo is reduced significantly in strain 19B grown in the presence of 3.5 μmoles of lysine and 70 μmoles of hydroxylysine when compared with that of the mutant grown in the presence of 3.5 μmoles of lysine alone. The effect is similar to that observed for high concentrations of lysine (1). In the in vivo system, homocitric acid-forming activity is feedback-inhibited by lysine and hydroxylysine to about the same extent.

Among the enzymes responsible for the conversion of α-aminoadipic acid to lysine, only the level of saccharopine reductase is reduced significantly by hydroxylysine, and the effect is similar to that observed with higher concentrations of lysine in the medium. Synthesis of α-aminoadipic acid reductase is relatively insensitive to hydroxylysine or lysine. Lysine apparently stimulates the level of saccharopine dehydrogenase, the last enzyme of the lysine pathway. However, hydroxylysine neither stimulates nor reduces this enzyme level.

It seems likely that, as with bacteria (6), hydroxylysine, mimicking lysine, controls the biosynthesis of lysine, and the inhibition of growth results from the inability of the organism to utilize hydroxylysine in the place of lysine. Unlike bacteria, in *Saccharomyces*, *N*-formyllysine exhibits no effect comparable to that of hydroxylysine.

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