ON THE MODE OF ACTION OF A PEPTIDE INHIBITOR OF GROWTH IN P. CEREVISIAE

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L-valyl-L-valyl-D-valine (Shankman and Schvo, 1958), is an effective (ID_{50}=20-30 μg/ml) inhibitor of growth in P. cerevisiae, ATCC #8081, and L. plantarum, ATCC #8014 (Shankman, Higa and Gold, 1960). Previous studies on the growth inhibition of P. cerevisiae by LLD val-val-val have revealed the following:

a. Inhibitory activity is dependent on both the type and the optical configuration of each amino acid residue in the tripeptide. Of the 8 possible stereo-isomers of trivaline, only the LLD val-val-va was active (Shankman, Higa and Gold, 1960; Shankman, Gold and Higa, 1961). Of the 8 possible tripeptides of the LLD configuration, containing valine and/or leucine, only the trivaline and valyl-valyl-leucine were significantly inhibitory (Shankman and Higa, 1962).

b. LLD val-val-val added to exponentially growing P. cerevisiae prolongs the doubling time but does not prevent eventual attainment of maximum growth.

c. LLD val-val-val is not significantly hydrolyzed in 24 hours at 30°C by heavy suspensions of P. cerevisiae. Trivaline of LLL configuration is completely hydrolyzed to free valine in 3 to 6 hours under these conditions.

d. LLD val-val-val inhibition is species specific. With the exception of L. plantarum, other lactic acid bacteria, as well as E. coli W

(both the wild type and a valineless mutant), S. faecalis, and S. aureus were not significantly inhibited (Shankman, Higa and Gold, 1960; Shankman, Gold and Higa, 1961).

Peptide Reversal of LLD Val-Val-Val Inhibition. A number of peptides have now been found to reverse LLD val-val-val inhibition of P. cerevisiae. Some of these are listed in Table I. Two points are of special interest: (1) Several reversers contain uncommon amino acid residues, and (2) Inhibition and competitive reversal are obtained, respectively, with LLD trivaline and LLD trivaline methyl ester, both of which are poorly utilized as valine sources by P. cerevisiae (Shankman, Higa, Florsheim, Schvo, and Gold, 1960).

TABLE I

Reversal of LLD Val-Val-Val Inhibition of P. cerevisiae Growth by Peptides and Amino Acids

Reverser	Concentration mg/ml	% Reversal[a]
gly-DL-norvaline	.100	61
gly-DL-α-amino butyric acid	.100	49
L-val-L-val-D-val methyl ester	.100	81
D-val-D-val-D-val methyl ester	.100	0
Peptone	2.0	91
Complete amino acid mix (P. cerevisiae)	4.0	4
	8.0	14
	12.0	57

LLD trivaline concentration 50 μg/ml. Inhibitions were at least 80%. Free amino acids were the sole source of amino acids in the unsupplemented medium. Medium composition and growth conditions have been described (Shankman, Higa, Florsheim, Schvo and Gold, 1960). Bacterial growth was measured turbidimetrically after incubation for 18 hours.

(a) $\%\ \text{reversal} = \frac{T(\text{reverser + inhibitor}) - T(\text{inhibitor alone})}{T(\text{reverser alone}) - T(\text{inhibitor alone})} \times 100$

T = turbidity (as optical density).

Peptone gave complete reversal at 2 mg/ml while only partial reversal was obtained by a complete mixture of amino acids at 12 mg/ml. The work of several investigators (Dunn, Ravel and Shive, 1956; Kihara and Snell, 1960; Leach and Snell, 1960; O'Barr and Pierce, 1960) has clearly established that, in several species of bacteria, independent uptake mechanisms exist for peptides and amino acids, and that, in some cases, an amino acid can be more efficiently supplied to the bacteria in peptide form. These facts led us to suspect that LLD trivaline may inhibit bacterial growth by interfering with the uptake of some amino acid(s), and that peptone may reverse the inhibition by "bypassing" the block in the uptake of the free amino acid, and/or by displacing LLD trivaline from its binding site.

Effect of LLD Val-Val-Val on Uptake of C^{14} Amino Acids. To determine the effect of LLD val-val-val on the uptake of amino acids by P. cerevisiae, washed bacteria were incubated in a medium containing potassium phosphate, glucose, and C^{14} amino acid. Chloramphenicol was added to prevent the incorporation of amino acids into protein without interfering with assimilation into the free amino acid pool (Leach and Snell, 1960; Hancock, 1960).

LLD trivaline causes a reduction in the binding of some amino acids while leaving others only slightly affected. In the presence of LLD trivaline, the binding of L-histidine, L-methionine, L-phenylalanine, L-tryptophan, L-leucine and L-valine by P. cerevisiae was significantly reduced, while DL-glutamic acid, DL-lysine, L-serine, L-alanine, DL-aspartic acid, and glycine were little affected. The reduction in binding caused by LLD trivaline is partially or completely reversed by LLD trivaline methyl ester (Table II).

The magnitude of the reduction in binding appears to depend on the kind of amino acid used and on the length of the incubation. Incubation periods ranging from 8 to 24 hours are required to produce the maximum effect. In the case of L-histidine this appears to be at least partly due to the slow uptake of the amino acid. Under the conditions of these experiments, pools of several of the amino acids are well maintained for at least 24 hours.

TABLE II

Effect of LLD trivaline and LLD trivaline methyl ester on Binding of C^{14} Amino Acids by P. cerevisiae

C^{14} Amino Acid	C.P.M. x 10^3 (Average of 2)		
	Control	+LLD val-val-val (100 µg/ml)	+LLD val-val-val (100 µg/ml) +LLD val-val-val methyl ester (200 µg/ml)
DL-glutamic Acid	8.52	7.57	8.25
L-histidine	3.50	1.54	2.91
DL-lysine	8.13	7.48	8.33
L-methionine	2.48	1.76	1.95
L-serine	1.43	1.53	1.54

Bacteria were washed twice with, and finally suspended in, cold .01 M Mg-succinate pH 6.2. The incubation mix contained the following in a final volume of 2.2 ml; 200-300 µg bacteria (dry weight); .02 µc C^{14} amino acid, specific activities 3-10 µc/µ moles; 4 mg D-glucose; 100 µ moles potassium phosphate pH 6.6; 200 µg chloramphenicol; 10 µ moles Mg-succinate. The mixture was incubated 22 to 23 hours at 23°C. Bacteria were then filtered onto millipore HA filters, and washed with 1.0 ml 0.01 M Mg-succinate buffer pH 6.2. The filters were glued to aluminum planchettes with rubber cement (Britten, Roberts and French, 1955), air dried, and counted in a Nuclear Chicago gas flow counter. Each sample was counted for at least 1,000 counts, at least three times. All experiments were performed in duplicate.

Uptake of C^{14} LLD. At an LLD trivaline concentration of 10^{-6} M a glucose dependent uptake is observed, although the binding capacity for the peptide is only about 10% of that obtained with an equivalent concentration of amino acid.

Several peptides were tested for their ability to prevent the uptake of LLD trivaline by P. cerevisiae (Table III). The LLL trivaline, and peptone, reversers of LLD growth inhibition, interfere with the binding of LLD trivaline. The failure of several other stereo-isomers of trivaline to prevent binding demonstrates the stereospecificity of the interaction. Free amino acids appear to stimulate binding.

TABLE III

Displacement of C^{14} L-valyl-L-valyl-D-valine from P. cerevisiae by Some Peptides

Peptide Added	Concentration mg/ml	% Control	C.P.M. Average of 2
None		100	422 ± 22
L-val-L-val-L-val	0.1	5	22 ± 2
D-val-L-val-L-val	0.1	120	506 ± 4
L-val-D-val-D-val	0.1	103	435 ± 5
D-val-D-val-D-val	0.1	102	432 ± 8
L-val-D-val-L-val	0.1	109	460 ± 12
L-val-L-leu-D-val	0.1	55	232 ± 10
Complete amino acid mix for P. cerevisiae	1.0	151	638 ± 9
Difco Bacto-peptone	1.0	29	123 ± 2

The unsupplemented incubation mixture contained in a final volume of 2.2 ml: 200 µg dry wt. bacteria; 20 µ moles Mg-succinate pH 6.2; 10 mg glucose; 200 µg chloramphenicol; 200 µg L-valine; C^{14} LLD val-val-val 8,500 C.P.M. (1.1 mµ moles). The mixture was incubated for 45 min. at 35°C, then placed in an ice bath. The experiment was performed in duplicate. Samples were prepared for counting as described in Table II.

Discussion. LLD trivaline inhibition of growth in P. cerevisiae can be reversed by several peptides, some of which are not utilized by the bacteria for growth. High concentrations of a complete mixture of free amino acids are required to give even partial reversal of growth inhibition under similar conditions. Radioactive LLD trivaline is displaced from P. cerevisiae by several peptides (some of which reverse LLD growth inhibition), but not by free amino acids in comparable concentration. LLD trivaline has been found to reduce the binding capacity of P. cerevisiae for several amino acids while leaving others unaffected. Two LLD trivaline effects, growth inhibition, and the reduction in binding of some amino acids, are reversed by LLD trivaline methyl ester.

These observations appear to be consistent with the following assumption

1. A structure associated with the active transport of amino acids has two classes of binding sites on its surface: one for amino acids, the other for peptides. The assumption of independent binding sites for peptides and amino acids is consistent with the findings of other workers (e.g. Kihara and Snell, 1960).
2. The binding of some amino acids is influenced by the binding of peptides at adjacent sites. It is conceivable that a reduction in affinity for an amino acid might be brought about by a change in the conformation of the whole binding structure due to interaction with a peptide. (Gerhart and Pardee, 1962).
3. LLD trivaline competes with LLD trivaline methyl ester and other peptides for a common binding site. LLD trivaline binding reduces the affinity of adjacent sites for some amino acids, while LLD trivaline methyl ester does not.

To our knowledge, little evidence has been reported previously to support the idea that small peptides influence the uptake of amino acids by bacteria. However, the observation (Demain and Hendlin, 1958) that histidine inhibition of growth in a B. subtilis mutant was reversed by a variety of glycine peptides might be interpreted in this way.

The model presented above bears some resemblance to the "allosteric" inhibition model (Monod and Jacob, 1961; Gerhart and Pardee, 1962), which was proposed to account for pseudo-competitive enzyme inhibition by molecular species structurally unrelated to the natural substrate. Enzymes subject to allosteric inhibition are thought to possess two separate, but interacting, binding sites, one for substrate, the other for inhibitor.

An investigation of the uptake kinetics of both LLD trivaline and amino acids is in progress.

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