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*CONTROL OF ENZYME ACTIVITY BY CONCERTED FEEDBACK INHIBITION**

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Recent studies[1-4] on end-product regulation in biosynthesis of amino acids of the aspartic family in microorganisms have revealed the existence of several alternative control patterns. The present report describes a phenomenon, observed in a photosynthetic bacterium, which provides a basic element for one control scheme, viz., feedback inhibition of the activity of the first enzyme (β-aspartokinase) in the branched biosynthetic pathway by the simultaneous action of two amino acid end products. It is considered likely that this type of effect, which we designate as *concerted feedback inhibition*, will prove to have a general significance, especially in branched or interconnecting pathways of biosynthesis.

Materials and Methods.—Growth of bacteria: The strain of *Rhodopseudomonas capsulatus* used was a new isolate, with properties closely corresponding to those described by van Niel.[5] It was grown photosynthetically in a synthetic medium identical to that specified by Ormerod *et al.*,[6] except that the nitrogen source was 0.1 per cent L-glutamate instead of ammonium sulfate, and thiamine hydrochloride (1 mg/l) was added in place of biotin. Cells were harvested during the logarithmic phase of growth for preparation of extracts.

Enzyme preparation: Extracts were prepared by sonic disruption (10-kc oscillator) of washed cells suspended in 0.05 M potassium phosphate + 0.02 M β-mercaptoethanol buffer pH 7.2, under an atmosphere of argon. The sonicate was clarified by centrifugation at 18,500 $\times$ g for 30 min, followed by a second centrifugation at 30,000 rpm for 16 hr (Spinco rotor no. 30). Saturated ammonium sulfate solution,

adjusted to pH 7.4 with concentrated ammonium hydroxide, was then added dropwise to the extract until 0.45 saturation was attained. The resulting protein precipitate was removed by centrifugation and the supernatant fluid was brought to 0.65 (or 0.70) saturation with respect to ammonium sulfate. The precipitate formed was collected by centrifugation and dissolved in the buffer noted to give a protein concentration of 10 mg/ml. Reprecipitation with ammonium sulfate between 0.31 and 0.50 saturation provided the fraction employed in the present experiments; the precipitate was dissolved in buffer and dialyzed against 100 vol of the same buffer for 20 hr. All of the foregoing operations were performed at 0–4°.

The preparation had a specific aspartokinase activity of 80 (see below) per mg protein, representing a purification of approximately 14-fold with respect to the crude sonic extract. Storage at 4°, at a protein concentration of 25.5 mg/ml, did not result in significant loss of activity over a period of several weeks. Just before assay, the enzyme solution was diluted, as required, with 0.05 *M* tris (hydroxymethyl)aminomethane-HCl buffer [tris] pH 7.2.

Enzyme assay: β-Aspartokinase activity was measured essentially as described by Black and Wright,[7] by estimating the quantity of asparthydroxamate formed in the presence of neutralized hydroxylamine. The reaction mixtures contained (in μmoles) the following components, in a final volume of 1 ml: tris pH 8.1, 95; L-aspartate, 10; adenosine 5′-triphosphate (ATP), 20; hydroxylamine, 600; $MgCl_2$, 20; KCl, 200; β-mercaptoethanol, 5; amino acid modifiers, as indicated; and appropriate amounts of enzyme. Following incubation for 30 min at 26°, the reaction was terminated by addition of 1 ml of $FeCl_3$ reagent.[8] Blank reaction mixtures containing all components except aspartate were always included as controls. Enzyme activity is expressed as 1000 × absorbancy, at 540 mμ, of the asparthydroxamate-iron complex; one unit of aspartokinase is defined as the amount of enzyme causing a change in absorbancy (1-cm light path) of 0.001. Under the assay conditions used, activity was linear with respect to enzyme concentration and time up to an absorbancy value of about 0.090.

Estimation of protein: Protein was determined either spectrophotometrically[9] or by the biuret method.[10]

Results and Discussion.—Concerted feedback inhibition by L-lysine and L-threonine: Figure 1 shows the effects of two products of the branched biosynthetic pathway on the aspartokinase activity. It can be seen (Fig. 1*A*) that lysine and threonine, individually, at 2 mM concentration do not significantly affect enzyme activity (however, see below). When both amino acid end products are present, enzyme activity is markedly depressed.

The results of Figure 1*B* sho v that with increasing concentration of one of the amino acids (L-lysine) at a constant level of the other (L-threonine), the extent of inhibition rises rapidly and reaches a maximum value. With 2 mM lysine and 1 mM threonine, the reduction in enzyme activity is more than 50 per cent, and at 4mM lysine, the maximal inhibition of approximately 75 per cent is attained. Similar results were observed in the reciprocal type of experiment, in which the lysine concentration (e.g., 2 mM) is held constant and the threonine concentration varied. Concerted feedback inhibition of aspartokinase activity by threonine + lysine was found to be nontotal even with the amino acids at 10 mM concentration,

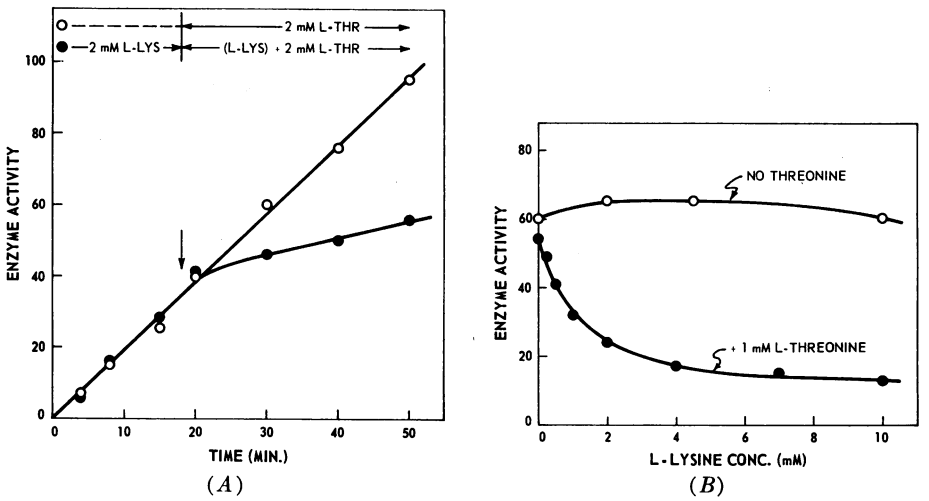

FIG. 1.—(*A*) Effects of L-lysine, L-threonine, and L-lysine + L-threonine on *Rps. capsulatus* aspartokinase activity. Two solutions, each containing 0.75 mg protein per ml of standard reaction mixture, were incubated with or without 2 mM lysine, as indicated, at 26°. At the times denoted by the points, 1-ml samples were removed and the quantities of asparthydroxamate estimated. At 18 min (arrow), both solutions were supplemented with L-threonine (2 mM). (*B*) Progressive inhibition of aspartokinase activity with increasing concentration of L-lysine at a constant level (1 mM) of L-threonine. The reaction mixtures each contained 0.75 mg protein and were supplemented with amino acids as indicated; standard assay conditions were employed.

using both (crude) sonic extracts and the partially purified enzyme. The residual activity may reflect enzyme "desensitized" with respect to feedback modifiers by the extraction procedures used or the presence of a second species of aspartokinase which is not subject to feedback inhibition control. The presence of a second enzyme, insensitive to concerted feedback inhibition, presumably would facilitate continued production (*in vivo*) of common intermediates required for methionine synthesis under conditions of lysine + threonine excess.[4]

It is noteworthy that lysine and threonine, individually, have a detectable influence on the aspartokinase activity (see Fig. 1*B*). With lysine, a slight stimulation is commonly observed, even at low concentration levels (e.g., see Figs. 1*B* and 2). Threonine, on the other hand, causes a slight (10–15%) inhibition of the enzymatic reaction (e.g., see Fig. 1*B*). Although these effects are small, they have been consistently observed in a number of experiments and may have significance in the over-all physiological regulation of aspartokinase activity.

Reversibility of concerted feedback inhibition: The data of Table 1 demonstrate that the concerted inhibition of aspartokinase activity by lysine + threonine is completely reversible. When the enzyme was preincubated with 2 mM lysine + 2 mM threonine (II) and subsequently diluted for assay (with lysine and threonine present at 0.1 mM concentration), full activity was observed. With 2 mM lysine + 2 mM threonine present during assay, activity of enzyme preincubated in the presence of lysine + threonine was severely inhibited.

"Regulatory sites" for lysine and threonine: The experimental evidence presented above indicates that although lysine and threonine individually influence the aspartokinase activity to a small extent, the presence of both end products is required for effective feedback inhibition. This observation, in itself, suggests that

TABLE 1

REVERSIBILITY OF CONCERTED FEEDBACK INHIBITION OF ASPARTOKINASE ACTIVITY

	During Preincubation (conc., mM) L-lysine	L-threonine	During Assay (conc., mM) L-lysine	L-threonine	Enzyme activity
I	None	None	None	None	77
	"	"	2.0	None	84
	"	"	None	2.0	73
	"	"	0.1	0.1	77
	"	"	2.0	2.0	24
II	2.0	2.0	0.1	0.1	78
	2.0	2.0	2.0	2.0	22
III	0.1	0.1	0.1	0.1	73
	0.1	0.1	2.0	2.0	19

Enzyme solutions (20.4 mg protein/ml, in 0.05 *M* potassium phosphate + 0.02 *M* β-mercaptoethanol buffer pH 7.2) were preincubated at 26° for 20 min, with or without amino acids, as indicated. The aspartokinase activity of 0.05 ml of each mixture was then measured using the routine assay procedure; final concentrations of amino acids present during assay are given in columns 4 and 5.

in addition to specific substrate (aspartate) sites, *Rps. capsulatus* aspartokinase possesses distinct "regulatory" sites for lysine and threonine. Support for this conclusion was obtained from experiments on protection of the enzyme by amino acids against heat inactivation, and from a preliminary kinetic analysis of concerted feedback inhibition.

Heating the enzyme at 43° for 7 min in the absence of amino acids resulted in loss of approximately 80 per cent of the activity (Table 2). L-lysine, L-threonine, and especially a combination of the two amino acids are effective in protecting the enzyme against heat inactivation. Similar protection of activity by feedback inhibitors has been reported for other biosynthetic enzymes.[11, 12] In contrast with the foregoing, D-threonine, L-aspartate, and DL-α, ε-diaminopimelic acid do not protect the *Rps. capsulatus* aspartokinase. These facts are interpreted as evidence for the existence of separate sites on the enzyme for L-lysine and L-threonine, which can be specifically occupied by the respective amino acid in the absence of the other.

The kinetic data presented in Figure 2 show that the concerted inhibition of aspartokinase activity by lysine + threonine is noncompetitive with respect to L-aspartate. This indicates that the "regulatory" sites for lysine and threonine are distinct from the aspartate (substrate) sites.

Specificity experiments: The aspartokinase activity is not affected by L-methionine or L-isoleucine, the other two end products of the branched pathway.[4] Similarly, the intermediates L-homoserine (5 mM) and DL-α, ε-diaminopimelic acid (8mM), individually, in combination with each other, or in combination with

TABLE 2

PROTECTION OF ASPARTOKINASE BY L-LYSINE AND L-THREONINE AGAINST HEAT INACTIVATION

Additions, before heating	Enzyme activity (% of unheated control)
None	21
L-lysine (1 mM)	51
L-threonine (1 mM)	59
L-lysine (1 mM) + L-threonine (1 mM)	70

Enzyme solutions (5.1 mg protein/ml, in 0.05 *M* potassium phosphate +0. 02 *M* β-mercaptoethanol buffer pH 7.2) were heated at 43° for 7 min in the presence or absence of amino acids, as noted. After heating, the mixtures were chilled rapidly, and the aspartokinase activity of 0.2 ml of each mixture was measured using the routine assay procedure (at 0.2 mM concentration, lysine and threonine do not influence enzyme activity appreciably). The unheated control was not supplemented with amino acid modifiers and was kept at 4° until assay.

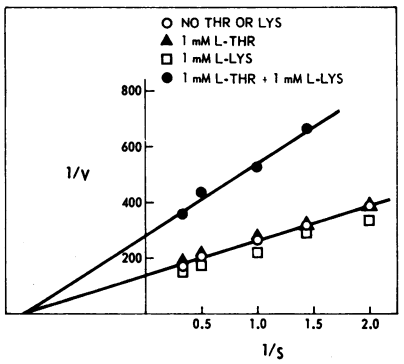

FIG. 2.—Noncompetitive inhibition of aspartokinase activity by L-lysine + L-threonine; double reciprocal plot of substrate concentration and initial reaction velocity. 1/S, reciprocal of L-aspartate concentration, in mmoles per liter; 1/v, reciprocal of initial reaction velocity, measured as amount of asparthydroxamate formed in 30 min and expressed as 1000 × absorbancy (at 540 mμ) of the asparthydroxamate-iron complex. The reaction mixtures each contained 0.75 mg protein and supplemental amino acids as noted.

either L-lysine or L-threonine, do not influence enzyme activity. The combination of L-lysine (1 mM) + D-threonine (1 mM) did not inhibit enzyme activity, but at higher concentrations of D-threonine appreciable inhibition was noted. Graphical analysis of inhibition curves obtained using a fixed concentration of L-threonine and varying concentrations of L-lysine or DL-lysine indicated that concerted feedback inhibition is not effected by the combination of D-lysine + L-threonine.

Physiological implications of concerted feedback inhibition: Oversynthesis of amino acids and various other "end products" in growing microbial cells is prevented, in part, by the phenomenon of feedback inhibition of enzyme activity.[13] Thus, in linear (unbranched) biosynthetic pathways, the end product characteristically inhibits the activity of an early enzyme in the sequence. Similar regulatory control occurs in branched biosynthetic pathways, in which a common precursor is converted through diverging metabolic sequences to several essential products. Assuming that the biosynthetic apparatus of the cell is geared for maximal economy, it is apparent that in addition to end-product control within *each* branch of such a pathway, regulatory mechanisms are required for controlling the *formation of common precursors* under conditions when certain products are present in "excess." In principle, one scheme for achieving such control could be based on specific feedback inhibition of an early enzyme by the concerted effect of two or more end products. The *in vitro* experimental results presented in this report support this prediction in that two end products of the aspartic family pathway, lysine and threonine, individually do not appreciably affect the activity of β-aspartokinase (the first enzyme) but, when present simultaneously, cause effective inhibition. The operation of concerted feedback inhibition of enzyme activity as a regulatory device may well be expected in other branched or interconnecting pathways of biosynthesis.

Summary.—The phenomenon of concerted feedback inhibition of enzyme activity is described. This type of feedback control depends on the simultaneous action of two end products of a branched biosynthetic pathway. The enzyme studied, viz., β-aspartokinase from the photosynthetic bacterium *Rhodopseudomonas capsulatus*, is virtually insensitive to individual end products. The specific combination of L-lysine + L-threonine, however, causes effective inhibition. Concerted inhibition due to these amino acids is nontotal, reversible, and noncompetitive with respect to one of the substrates of the enzymatic reaction, namely, L-aspartate. Evidence is presented for the conclusion that the β-aspartokinase pos-

sesses separate "regulatory sites" for L-lysine and L-threonine, which are distinct from the substrate (L-aspartate) sites. The probable role of concerted feedback inhibition in regulation of synthesis of aspartic family amino acids (lysine, threonine, isoleucine, methionine) in *Rps. capsulatus*, and in branched pathways in general, is discussed.

Note added in proof: After this manuscript went to press, a report [Caskey, C. T., D. M. Ashton, and J. B. Wyngaarden, *J. Biol. Chem.*, **239**, 2570 (1964)] appeared describing synergistic effects of pairs of feedback inhibitors on the activity of pigeon liver glutamine phosphoribosylpyrophosphate amidotransferase; the data reported show elements of similarity with the phenomenon of concerted feedback inhibition.

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THE SATELLITE TOBACCO NECROSIS VIRUS: A SINGLE PROTEIN AND ITS GENETIC CODE

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The Rothamsted culture of tobacco necrosis virus was reported to consist of three components, distinguishable by their sedimentation coefficients of 50s, 122s, and 222s, respectively.[1] When the components were separated on sucrose density gradient columns and examined with the electron microscope, two types of polyhedral particles were observed. The two light components had diameters of 20 and 28 mμ, respectively, while the heavy component was found to be a rosette of twelve 20-mμ particles. When the 20-mμ particles were inoculated by themselves into host plants, they were shown to be unable to multiply in the absence of the 28-mμ particles. However, the 28-mμ particles, inoculated by themselves into host plants, produced the typical necrotic lesions from which only 28-mμ particles could be isolated. Serological studies showed that the 28-mμ particles were related