Regulation of Branched Biosynthetic Pathways in Bacteria

Studies of the key enzymes in various organisms reveal multiple metabolic feedback patterns.

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Repression of enzyme synthesis and end-product inhibition of enzyme activity are two basic mechanisms for control of metabolic reactions in biological systems (*1*). These negative feedback devices (*2*), often mutually nonexclusive, are designed to adjust the rates of synthesis of cellular metabolites by regulating the concentrations or activities, or both, of certain key enzymes required for the synthesis of these end products. In a linear biosynthetic pathway the first enzyme of the sequence appears to be an effective control point (*3*); regulatory control of this step is all that is required to ensure balanced production of the end product. In a branched biosynthetic pathway in which a common precursor gives rise to several end-product metabolites through a series of enzymatic reactions, however, a high degree of complexity is to be expected, especially in regard to the control of the early enzymes of the pathway. The overall regulatory plans of such pathways depend largely on the nature of the biochemical reactions, the extent of branching, as well as the physiological requirements of the given organism. Considering the genetic differences and metabolic diversity among various species, it is not surprising therefore that, even within a given biochemical pathway, considerable variation in the overall control pattern is a rule rather than an exception. The control of biosynthesis of the amino acids of the aspartate family is a particularly suitable example to illustrate this point. In this branched pathway, end-product metabolites, individually or in various combinations, critically influence several key enzymatic reactions. My purpose here is to summarize these negative feedback devices that have been recently uncovered and to delineate alternative overall regulatory schemes for the control of biosynthesis of four amino acids from a single common precursor aspartate. I have also attempted to show that, at least in this pathway, common enzymatic steps and similar control patterns observed in various bacterial species do not necessarily reflect evolutionary affinities of the structural genes coding for the proteins which are involved in catalysis and control of these catalytic reactions.

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Aspartate Pathway

In bacteria, aspartic acid is the common precursor for the synthesis of several important amino acids, namely, lysine, methionine, threonine, and isoleucine (Fig. 1) (*4*). In the initial step aspartic acid is phosphorylated to aspartyl β-phosphate by the enzyme aspartokinase (E.C.2.7.2.4); the reaction requires adenosine triphosphate (ATP) and a divalent cation. The phosphorylated product is then dephosphorylated and subsequently reduced to yield aspartate β-semialdehyde. Both these reactions are presumably carried out by the enzyme aspartate β-semialdehyde dehydrogenase (E.C.1.2.1.11) in the presence of a reduced pyridine nucleotide. The third enzyme in the sequence, homoserine dehydrogenase (E.C.1.1.1.3), which is also a pyridine nucleotide-linked enzyme, transforms aspartate β-semialdehyde to homoserine, the latter amino acid serving as a precursor for the synthesis of the three amino acids, methionine, threonine, and isoleucine. Aspartate β-semialdehyde is also a starting product required for lysine biosynthesis; condensation of aspartate β-semialdehyde with pyruvate yields dihydrodipicolinic acid, which is converted to lysine through a linear sequence of at least six enzymatic steps (*4*).

For the synthesis of methionine from homoserine, cysteine is required for formation of the thioether linkage. Homoserine is initially succinylated by homoserine *O*-transsuccinylase in the presence of succinate, ATP, and coenzyme A, and the product, *O*-succinylhomoserine, reacts with cysteine to give cystathionine. Hydrolysis of the thioether linkage of cystathionine and subsequent methylation of the sulfhydryl group yields methionine. It should be pointed out that cysteine per se is not a direct intermediate in bacterial methionine biosynthesis, but rather is the terminal metabolite of a separate biosynthetic pathway; cysteine donates its three-carbon unit for the transsulfuration reaction resulting in cystathionine synthesis.

From the biosynthetic scheme shown in Fig. 1, it is apparent that the first two enzymes, aspartokinase and aspartate β-semialdehyde dehydrogenase, are common for the synthesis of four amino acids, whereas the third enzyme in the sequence, homoserine dehydrogenase, is shared for the synthesis of three amino acid end products. The remaining reactions can be designated as terminal branches which are linear in that each branch is unique for the synthesis of one end product only. For example, dihydrodipicolinic acid, *O*-succinylhomoserine, and α-ketobutyric acid may be denoted as the initial precursors of the lysine, methionine, and isoleucine branches, respectively. Since threonine is an "end product" for protein synthesis as well as an intermediate in isoleucine biosynthesis, two enzymes—namely, homoserine kinase (E.C.2.7.1.39) and threonine synthase (E.C.4.2.99.2)—are usually referred to as part of the threonine branch.

It is evident from the summary scheme (Fig. 1) that aspartate β-semialdehyde and homoserine are crucial intermediates of the aspartate pathway. The cellular concentrations of these two precursors must be maintained at their proper levels if the syntheses of these amino acids are to be balanced according to the exacting physiological requirements of the organism. In the

event that a particular end product is in excess of its normal concentration, we can envisage that the rate of synthesis may be temporarily reduced by controlling the first step of that linear sequence involved in the synthesis of the end product in question; for example, the synthesis of lysine can be effectively regulated by reducing the rate of synthesis of dihydrodipicolinic acid from aspartate β-semialdehyde (*5*). These feedback mechanisms (both repression and end-product inhibition) will have very little effect on the synthesis of the other amino acids. Experiments with other key branch-point enzymes reveal that such control mechanisms are indeed commonly observed for the individual branches for the synthesis of lysine, methionine, threonine, and isoleucine (Fig. 1) (*1*).

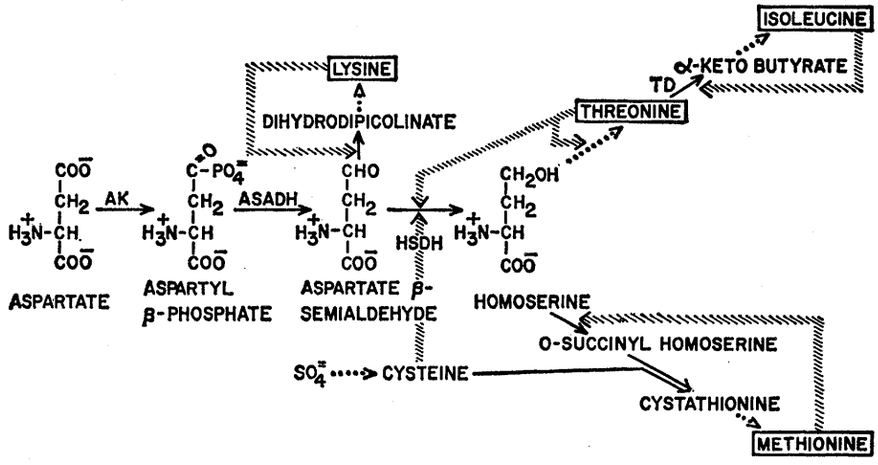

Fig. 1. Aspartate pathway showing regulation of the individual terminal branches and branch-point enzymes (hatched lines). Broken arrows indicate more than one enzymatic step. Abbreviations are: AK, aspartokinase; ASADH, aspartate β-semialdehyde dehydrogenase; HSDH, homoserine dehydrogenase; TD, threonine deaminase.

If we assume that these results of studies in vitro can be justifiably translated to situations in vivo, the important question, nevertheless, is what are the mechanisms for controlling the earlier reactions of the aspartate pathway that are common for the synthesis of the amino acid end products? There is no unique mechanism for the regulation of aspartokinase and homoserine dehydrogenase in various bacteria (*6*, *7*), but several alternative overall control patterns have been uncovered for the control of synthesis of aspartate β-semialdehyde and homoserine. Depending on the precise regulatory plan, a single modifier or more than one modifier is adequate to regulate the proper flow of metabolites from aspartate to homoserine. The first three patterns, namely, isoenzymic control, sequential feedback, and linked-pathway control depict the effect of a single modifier on the individual enzymatic steps; the last three patterns including concerted feedback, compensatory feedback, and multivalent repression operate when more than one end product is responsible for the regulation of a given biochemical reaction by influencing the concentration or activity, or both, of the enzyme essential for that particular step.

Isoenzymic Control

In a branched pathway (Fig. 1), regulation of aspartokinase by lysine, for example, should limit the synthesis of aspartate β-semialdehyde (and thus homoserine) required for the synthesis of methionine and threonine; control by threonine, similarly, would result in the reduction of the amount of aspartate β-semialdehyde essential for lysine and methionine biosynthesis. These restrictions, however, would be eliminated if there were three separate isoenzymes of aspartokinase, each one under the regulatory control of a single amino acid end product. In *Escherichia coli* (*8*, *9*) and *Salmonella typhimurium* (*10*) it has indeed been found that both aspartokinase and homoserine dehydrogenase are present in multiple forms. Stadtman, Cohen, and their collaborators (*8*, *9*) have isolated and identified three distinct aspartokinases in *E. coli*—namely, threonine-sensitive aspartokinase (AKI), methionine-repressible kinase (AKII), and a third enzyme specifically inhibited by lysine (AKIII); the latter enzyme is also repressible by lysine. These enzymes have been separately purified, and their properties have been studied (*9*). For the purpose of our discussion it is sufficient to know that the specificity of modifier effects with respect to these enzymes are absolute. For example, AKI is inhibitable only by threonine, and AKIII is under the end-product control of lysine. The methionine-repressible enzyme, AKII, is present in very small amounts in the extracts of wild-type *E. coli*. It would appear from these findings that the control of each of these isoenzymes by any single end product is analogous to the type of controls observed in a linear biosynthetic pathway. In other words, it may be argued that the individual pathways involved in the biosynthesis of all these end products begin with aspartic acid, the common starting precursor of all amino acids (and not from the respective branch points), and the earlier reactions, although catalytically indistinguishable, are indeed three separate branches running parallel to each other.

According to the foregoing interpretation one would expect only two homoserine dehydrogenases, one regulated by methionine, the second by threonine. The presence of a third enzyme subject to feedback controls by lysine is not essential because homoserine is not a precursor for lysine (Fig. 1). The work of Cohen, Patte, and their co-workers with *E. coli* (*9*), and the observation reported by Freundlich with *S. typhimurium* (*10*) have indeed shown that in these organisms there are two homoserine dehydrogenases, HSDH I and HSDH II, controlled by the end products threonine and methionine, respectively. These enzymes are separable from one another, and they are sufficiently different to be recognized as two distinct protein species (*9*, *10*).

Genetic evidence, as well as biochemical studies, has indicated that the catalytic activities and regulatory potentials of AKI and HSDH I are part of a single protein or protein complex; activities of AKII and HSDH II may also reside in a single protein (*9*). For example, a single mutational event abolishes both AKI and HSDH I activities, and revertants of this mutant regain both activities simultaneously. Furthermore, homoserine and reduced nicotinamide-adenine dinucleotide (NADH), which are substrates for

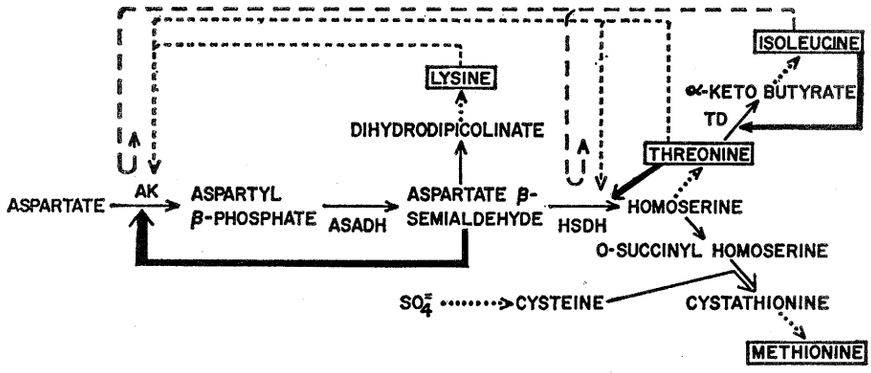

Fig. 2. Aspartate pathway showing sequential feedback inhibition (solid lines) and compensatory feedback control (broken lines). See Fig. 1 for the controls of the individual terminal branches.

the homoserine dehydrogenase reaction, strongly influence the activity and stability of aspartokinase. Similar effects have been observed with the substrates of aspartokinase on the stability and activity of homoserine dehydrogenase (*11*). The significance of these "bifunctional" enzymes or enzyme-aggregates is not completely understood (*12*), although aggregation to form a complex of more than one gene product belonging to the same operon so as to elicit a single function (*13*) or multiple functions (*14*) has been observed in the tryptophan pathway and in many other systems (*15*). The existence of a single protein in *E. coli* with aspartokinase and homoserine dehydrogenase activities and control of such activities by common modifiers are certainly indicative of the efficiency of the catalytic as well as the regulatory properties of the two enzymic reactions. Before the complex aggregates of aspartokinase and homoserine dehydrogenase become known, Cowie (*16*) proposed an extensive model of a linked enzyme system in *E. coli* for the synthesis of the amino acids of the aspartate family.

Sequential Feedback Inhibition

An alternative to the isoenzymic pattern for the control of early enzymes of a branched pathway is inhibition by sequential feedback (*17*). In this case the end product controls the first enzyme of the terminal branch, with resulting accumulation of the branch-point intermediate; an excess amount of the intermediate in turn, regulates the earlier enzymatic steps. In *Rhodopseudomonas spheroides* the activity of aspartokinase is strongly inhibited by aspartate β-semialdehyde (*18*), while the homoserine dehydrogenase activity is inhibited by threonine (*19*) (Fig. 2). In this organism the sequence of events leading to the control of an early enzyme is as follows (Fig. 2). Overproduction of isoleucine inhibits threonine deaminase (E.C.4.2.1.16) activity (*20*), and accumulated threonine reduces the rate of homoserine synthesis by inhibiting the activity of homoserine dehydrogenase. This negative cascading effect tends to increase the intracellular concentration of aspartate β-semialdehyde, which, in turn, slows down its own production by decelerating the formation of aspartyl β-phosphate from aspartate through feedback inhibition control of aspartokinase (*18*). According to this interpretation, inhibition by threonine and by aspartate β-semialdehyde of the activities of homoserine dehydrogenase and aspartokinase, respectively, depletes the cells of their necessary lysine and methionine pools. This is not expected, however, because threonine and aspartate β-semialdehyde are competitive inhibitors with respect to the substrates aspartate β-semialdehyde, aspartate, and ATP, respectively (*18, 19*). Thus the concentration of aspartate β-semialdehyde may not fall below a "critical" level; presumably, this concentration will suffice for the synthesis of lysine and methionine. In addition to the illustrated sequential feedback, control by repression of aspartokinase synthesis and homoserine dehydrogenase synthesis by methionine has been observed. With auxotrophic strains of *R. spheroides*, both aspartokinase and homoserine dehydrogenase can be derepressed almost twofold by limiting the supply of methionine in the growth medium (Table 1).

Linked-Pathway Control

Since homoserine is the branch-point precursor for both methionine and threonine, end-product regulation of homoserine dehydrogenase by both methionine and threonine would provide useful means of adjusting the rates of homoserine synthesis. In various bacteria that have been examined, threonine is, in fact, an effective feedback inhibitor; however, the activities of homoserine dehydrogenase as well as two earlier enzymes, aspartokinase and aspartate β-semialdehyde dehydrogenase, are not subject to feedback inhibition by methionine (*21*). In most bacteria, methionine represses the synthesis of both homoserine dehydrogenase and aspartokinase (*6, 7*; also see Table 1). In view of this, a slow repression control of the enzyme synthesis seemed to be adequate to insure proper concentrations of intracellular methionine (*22*). I have reported that cysteine is a strong feedback inhibitor of several bacterial homoserine hydrogenases including that from *E. coli* K12 and from some members of the nonsulfur purple photosynthetic bacteria (*23*). The effect is stereospecific for L-cysteine; two other intermediates of the methionine branch, cystathionine and homocysteine, have no significant effect. The cysteine inhibition of homoserine dehydrogenase activity suggests the following rationale for the control of homoserine biosynthesis. In the event that the cysteine pool in the cell is increased, synthesis of cystathionine (and therefore methionine) may be enhanced if sufficient amounts of homoserine were available. Overproduction of methionine may be prevented by end-product control (repression and feedback inhibition by methionine itself) of the enzymes unique for the methionine branch (*7*) or by regulating the homoserine concentration in the cell by decreasing the rate of homoserine synthesis through inhibition by cysteine of homoserine dehydrogenase activity, or both (*23*). Similarly, overproduction of threonine is prevented by analogous feedback controls by this amino acid on homoserine dehydrogenase and homoserine kinase. In other words, the size of the intracellular homoserine pool depends on the concentrations of both cysteine and threonine in the cell (Fig. 1). In this pattern, the terminal metabolite of one pathway plays a dual regulatory role by controlling the activity of enzymes involved in its own

biosynthesis and by functioning as a regulatory modifier of a critical enzyme of a linked, or interconnecting, pathway (*23*). Nester (*24*) has reported that in *Bacillus subtilis* histidine can affect the synthesis and activity of certain enzymes of aromatic amino acid biosynthesis—a truly interesting example of cross-pathway regulation.

Concerted Feedback Inhibition

From the foregoing examples it is clear that, in addition to end-product control within each branch, regulatory mechanisms exist for controlling the formation of common precursors required for the synthesis of several end-product metabolites. In all these instances a safeguard must exist for the maintenance of an adequate cellular pool of intermediary metabolites after the block to ensure normal synthesis of the remaining end products which might otherwise be limiting. A particularly suitable example in the aspartate pathway is the simultaneous requirement of two end products for the regulation of activity of an early enzyme; this type of control has been variously referred to as concerted feedback inhibition (*6, 25*) or multivalent feedback inhibition (*26*). The aspartokinase activity of *Rhodopseudomonas capsulata* (*27*) is not influenced by any single end product of the branched pathway leading to the synthesis of lysine, methionine, threonine, and isoleucine (Fig. 1); however, when lysine and threonine are present simultaneously, the enzyme activity is severely inhibited (Fig. 3) (*25*). At 1 millimolar concentration each of L-lysine and L-threonine, a 50 percent reduction in activity is observed. The inhibition is specific for the lysine-plus-threonine combination, and is nontotal and noncompetitive with respect to aspartate (*25*). A similar inhibition of aspartokinase activity by the concerted action of these amino acids has been reported in *Bacillus polymyxa* and several other species of the genus *Bacillus* (*26, 28*). In contrast to *R. capsulata*, however, the activity of aspartokinase from *B. polymyxa*, as well as that from other *Bacillus* species, is inhibited to a significant extent when a large excess of either threonine or lysine was present.

The results of the studies in vitro on the concerted feedback inhibition show that, in the presence of excess lysine-plus-threonine, the activity of the *R.*

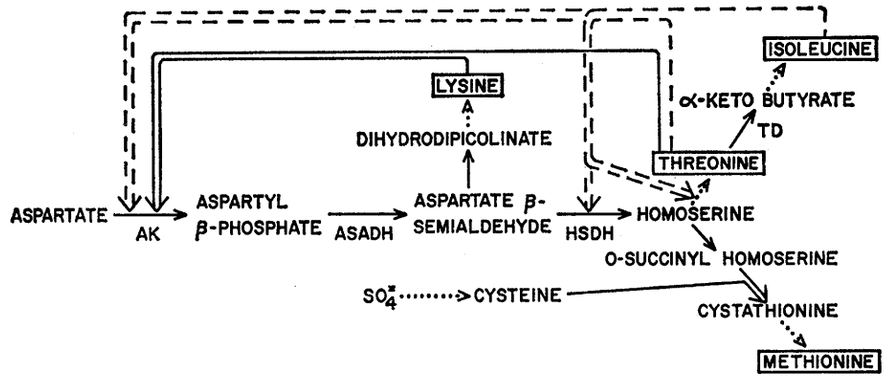

Fig. 3. Aspartate pathway showing concerted feedback inhibition (solid lines) and multivalent repression (dashed lines). See Fig. 1 for the controls of the individual terminal branches.

capsulata enzyme in crude as well as in partially purified enzyme preparations can be inhibited by as much as 75 percent (*25*). If we assume that the residual activity which is insensitive to concerted feedback inhibition is not due to desensitization but in fact is due to a second species of enzyme, continued production of aspartyl β-phosphate and other intermediates (required for methionine synthesis) will proceed even in the presence of excess amounts of lysine and threonine. Burlant *et al.* have reported that in *R. capsulata* methionine reduces the rate of synthesis of aspartokinase by one-half (*22*). It is as yet unknown whether the methionine-repressible enzyme and the enzyme inhibited by the lysine-plus-threonine combination are two distinct species of aspartokinase.

Evidence for the operation in vivo of concerted feedback inhibition as a regulatory device has been obtained from growth experiments (*22*). Growth of *R. capsulata* is severely inhibited when both lysine and threonine are included in the basal growth medium; further supplementation of the medium with methionine relieves the inhibition. In fact, the specific activity of aspartokinase from cells grown slowly in concentrations of lysine-plus-threonine which are not totally inhibitory did not differ significantly from that of cells grown in minimal medium containing no lysine and threonine. This comparison indicates that the control exerted by these amino acids on aspartyl β-phosphate production occurs through concerted feedback inhibition due to the presence of lysine-plus-threonine and not by a repression mechanism analogous to multivalent repression (*29*).

The simultaneous requirement for lysine-plus-threonine for the regulation of aspartokinase is not absolute. In

Table 1. Repression of *R. spheroides* aspartokinase and homoserine dehydrogenase by methionine (*45*). Organisms were grown in the synthetic malate plus glutamate medium (*18*), supplemented with amino acids as specified. Cell-free extracts were prepared by sonic disruption of cells in 0.05*M* potassium phosphate buffer at *p*H 7.5 containing 0.001*M* dithiothreitol, and clarified by centrifugation at 48,000*g* for 30 minutes. Just before assay, the enzyme solutions were passed through a Sephadex G-25 column equilibrated with the phosphate buffer. The activities of aspartokinase (AK), homoserine dehydrogenase (HSDH), and threonine deaminase (TD) were measured as described (*18, 20, 32*). Enzyme activities are expressed as follows: AK, micromoles of aspartohydroxamate formed per minute at 15°C; HSDH, change in absorbance at 340 nm per minute at 25°C; TD, micromoles of ketoacid formed per minute at 25°C. Specific activities are units per milligram of protein (*6*). The data show that the specific activities of aspartokinase and homoserine dehydrogenase decreased by about one-half when the growth medium contained excess L-methionine, whereas the specific activity of threonine deaminase remained unaffected under this condition. Met, methionine; Leu, leucine. Organisms having the genotype Met⁻Leu⁻ require these amino acids for their growth, whereas organisms with Met⁻ genotype require only methionine.

Strain	Genotype	Amino acid supplement (μg/ml)	Activities (units $\times 10^3$ per mg protein)		
			AK	HSDH	TD
M 29.5	Met⁻Leu⁻	Met 50; Leu 50	19	26	15
M 29.5	Met⁻Leu⁻	Met 2; Leu 2	35	42	16
Sp 1	Met⁻	Met 50	15	27	17
Sp 1	Met⁻	Met 10	29	46	17
Sp 1	Met⁻	Met 2	29	63	14

Pseudomonas putida for example, in addition to concerted feedback by the lysine-plus-threonine combination, the enzyme activity can also be drastically inhibited by the methionine-plus-threonine combination. In this organism threonine alone inhibits the aspartokinase activity, whereas methionine by itself has a stimulatory effect; when present together the inhibition observed is much greater than that found with threonine alone (*30*).

Compensatory Feedback Control

A unique pattern of control in the aspartate pathway by end products of opposing influence has been revealed in the photosynthetic bacterium *Rhodospirillum rubrum*. In this organism the activity of homoserine dehydrogenase is strongly inhibited by the feedback inhibitor threonine; the inhibition is not complete and is apparently noncompetitive with respect to aspartate β-semialdehyde (*31, 32*). Studies in vitro indicate that the threonine inhibition of the dehydrogenase activity can be reversed either by isoleucine or by methionine, the two end products of the pathway. In the absence of threonine, the latter amino acids stimulate the enzyme activity when assayed in the reverse direction, that is, for the oxidation of homoserine to aspartate β-semialdehyde (*32*); the physiological implications, if any, of the stimulatory effects are not yet known.

The regulatory pattern of aspartokinase from *R. rubrum* is more complex. Earlier results with cell-free extracts indicated that threonine inhibits the enzyme activity completely and that such inhibition could be reversed by isoleucine but not by methionine (*6*). More recent investigation (*30*) with partially purified enzyme preparations has revealed that, although threonine is a strong inhibitor of the enzyme activity, the inhibition is not complete even at very high concentrations of the amino acid; isoleucine and methionine individually reversed the threonine inhibition. Furthermore we have found that the aspartokinase activity is subject to concerted feedback inhibition by lysine and threonine (*30*). At low concentrations of lysine-plus-threonine, the inhibition is severe; individually these amino acids have very little effect at these concentrations. The most important aspect of the concerted feedback inhibition of aspartokinase in *R. rubrum* is that isoleucine and methionine can reverse the inhibition due to lysine-plus-threonine. No such reversal of concerted feedback has been observed with other bacterial enzymes including those from *R. capsulata* (*25*) and certain *Bacillus* species (*26, 28*).

In addition to the early enzymes of the aspartate pathway, threonine deaminase of *R. rubrum* (the first enzyme of the isoleucine branch) reveals some unusual properties. The activity of the *R. rubrum* enzyme, in contrast to biosynthetic deaminases studied thus far, is essentially insensitive to feedback inhibition control by isoleucine (*33*). When concentrations of substrate are low, minimum inhibition is observed in the presence of a large excess of the end product. Ning and Gest have reported (*34*) that a limited proteolytic digestion appears to sensitize the enzyme somewhat toward isoleucine inhibition. Nevertheless, it is clear that, in contrast to other bacterial species, the threonine deaminase step in *R. rubrum* cannot be considered as a focal control point for the regulation of isoleucine biosynthesis.

In view of the foregoing observations it is apparent that in *R. rubrum* the control of biosynthesis of amino acids of the aspartate pathway is achieved through a novel regulatory pattern different from the patterns outlined above. In this bacterium a temporal accumulation of threonine in the cell would cause a decrease in the concentrations of aspartyl β-phosphate and homoserine through feedback inhibition control of aspartokinase and homoserine dehydrogenase, respectively, thus preventing an adequate supply of lysine and methionine for protein synthesis. Excessive amounts of lysine-plus-threonine will similarly decrease the rate of methionine synthesis by the concerted feedback inhibition of aspartokinase activity. The synthesis of isoleucine from threonine would presumably continue at the normal rate, due to the lack of effective control at the threonine deaminase step. Since the inhibition by threonine or by the threonine-plus-lysine combination, or both, of homoserine dehydrogenase and aspartokinase activities is readily reversed by isoleucine, an increase in the ratio of isoleucine to threonine in the cell would trigger a metabolic signal for increasing the rate of synthesis of the common intermediates aspartate β-semialdehyde and homoserine for synthesis of lysine and methionine. Considering the biochemical relations for the synthesis of these amino acids, the overall control pattern observed in *R. rubrum* can be briefly described as the compensatory feedback control (Fig. 2) in which the feedback inhibition control by end-product threonine and by the threonine-plus-lysine combination can be compensated by an end product having an opposing effect (isoleucine) (*35*). Similar phenomena have been observed with several enzymes of various other biosynthetic pathways (*36*).

Results of in vivo experiments (*31*) on the effects of amino acid supplementation on the growth of *R. rubrum* are consistent with the in vitro data obtained with isolated enzymes. Growth of this organism is completely inhibited by 3.3×10^{-4} molar L-threonine, presumably because of interference with lysine and methionine synthesis; this inhibition may be reversed by further addition of L-isoleucine (3.3×10^{-4} molar).

Multivalent Repression

In addition to the feedback inhibition control of enzyme activity by more than one end-product metabolite, synthesis of certain biosynthetic enzymes can also be repressed by simultaneous presence of several end products. In *E. coli* and *S. typhimurium*, the formation of aspartokinase, homoserine dehydrogenase, and threonine synthetase is repressed by the threonine-plus-isoleucine combination (Fig. 3) (*29*). If one of these two amino acids is limiting, all three enzymes are derepressed, although the extent of derepression is not similar for each of these enzymes, an indication of lack of coordinate control. The advantages of multivalent repression are generally similar to those of concerted feedback inhibition; that is, in addition to the specific controls of synthesis of enzymes of the terminal branch by the end products, these mechanisms provide an additional means to insure against overproduction of metabolites by reducing the rates of synthesis of earlier enzymes. It is important to note that the threonine-plus-isoleucine combination affects only the threonine-sensitive species of aspartokinase and homoserine dehydrogenase (*9*).

Physiological Implications of Alternative Regulatory Schemes

From the above examples of alternative control patterns some general comments can be made in regard to the biosynthesis of amino acids of the aspartate family. In all bacterial species examined thus far, the activity of homoserine dehydrogenase has been found to be under feedback inhibition control of threonine (*37*) and cysteine (*23*), although important species differences exist between the actual mechanisms of modifier effects at the molecular level. The enzyme is also subject to repression by methionine and other end products. From the limited data available on the control of aspartate β-semialdehyde dehydrogenase (*38*), it would appear that this enzyme is not a focal control point in homoserine biosynthesis. With aspartokinase however, a large number of variations have been observed in regard to its control pattern.

In the enteric group of bacteria such as *E. coli* and *S. typhimurium*, isoenzymic patterns seem to be the rule; in the *Bacillus* species, with the exception of *B. licheniformis* where sequential feedback inhibition has also been reported (*28*), the dominant pattern is that of concerted feedback inhibition. With the nonsulfur purple photosynthetic bacteria (*Rhodospirillum* and *Rhodopseudomonas* species), even within a single genus, distinct regulatory schemes, including concerted feedback, sequential feedback, and compensatory feedback controls, have been uncovered. Whatever the overall regulatory pattern may be it is reasonable to assume that each pattern is adequate for a normal flow of metabolites from aspartate to various amino acid end products, and that each scheme is peculiarly suited to the physiology and metabolism of the organism. In this context it is important to note that, in the control of aromatic amino acid biosynthesis, the pattern of isoenzymic control of the first enzyme has been detected in *Escherichia* (*39*) and *Salmonella* species (*40*) whereas the *Bacillus* species reveal the dominant pattern of sequential feedback control (*40*). It is curious that in two distinct groups of organism, two independent biosynthetic pathways —one for aromatic amino acid and the other for amino acids of the aspartic family—exhibit somewhat similar control patterns. It is reasonable to expect, however, that if a given control pattern is indeed a highly efficient one, it may be found in a variety of biosynthetic pathways.

Evolutionary Significance

It has often been argued that the similarity in a given biochemical pathway or the mode of control of such a pathway may be useful to assess the phylogenetic relations in the context of biological evolution. For example, Vogel (*41*) has proposed two distinct evolutionary origins of lysine biosynthetic pathway, one proceeding through diaminopimelic acid and a second one through the α-amino adipic acid as the intermediate. Canovas, Ornston, and Stanier on the other hand, would prefer to believe that the "evolutionary significance of the presence of a given pathway in representatives of several different biological groups can be assessed by a somewhat different kind of analysis—comparison of control mechanisms" (*42*).

As far as it is known, in the aspartate pathway the transformation of aspartic acid to homoserine is mediated by three enzymes—namely, aspartokinase, aspartate β-semialdehyde dehydrogenase, and homoserine dehydrogenase (*4*). Accordingly, the aspartate pathway in various bacteria may have a single evolutionary origin. Divergent control patterns of these enzymes, especially of the first enzyme aspartokinase, however, may be taken as evidence for separate evolutionary origins. If we were to assume that common control mechanisms are indicative of evolutionary affinities and that control characteristics of a given biochemical pathway might be genetically conserved, one would predict that within a single genus *Rhodopseudomonas,* two species, *R. capsulata* and *R. spheroides*, which are morphologically and physiologically closely related but exhibit divergent control patterns (concerted feedback and sequential feedback, respectively) are two separate end products of evolution. On the other hand, the various *Bacillus* species and *R. capsulata* that have common control mechanisms for the regulation of homoserine biosynthesis may have evolved from a single origin (*43*).

Some properties of the aspartokinases of *R. capsulata* and *R. spheroides*, in fact, reveal that these proteins may be quite similar with respect to the modifier-binding sites—a property which is an essential prerequisite for the acquisition of a given control characteristic. Although the activities of the enzyme of *R. capsulata* and *R. spheroides* are inhibited by the lysine-plus-threonine combination and by aspartate β-semialdehyde, respectively, the *R. spheroides* aspartokinase, in addition to its ability to bind aspartate β-semialdehyde, has recognition sites for binding both lysine and threonine. This is judged by the protection provided by these amino acids against heat inactivation of this enzyme (see *18*).

This property may suggest that the aspartokinase protein was initially "committed" to the concerted type of control, but during evolution it had lost the regulatory effects of these amino acids (even though they are still able to bind) and a new modifier aspartate β-semialdehyde has evolved to control its function. The mutational change required for this newly acquired property might be a relatively simple one compared to those demanded for the *de novo* evolution of a completely new structural gene for the synthesis of a new protein with the added complexity of regulation by sequential feedback rather than by a concerted feedback mechanism. In other words, a mutational alteration of the modifier-binding sites of a given enzyme may conceivably lead to altered control characteristics without drastically changing the remaining primary sequence required for catalytic function. Furthermore it must be pointed out that, depending on the conformational states, a given protein may or may not be able to bind small modifier molecules required for the modulation of biological activity. If this were to be the case, diversity in control mechanisms may not indicate independent phylogenetic relationships. Therefore, to justify proposing a single evolutionary origin of a complex metabolic pathway, it is not sufficient to demonstrate either common catalytic steps or common control characteristics that regulate these enzymatic reactions. Homologous amino acid sequences between two or more proteins thus may yet prove to be the most crucial, though most difficult to obtain, evidence for comparison of evolutionary affinities (*44*).

Summary

Some complex interdependent regulatory interactions of end-product metabolites on the synthesis and activities of various enzymes involved in the biosynthesis of these end products are described. Analysis of the experimental data with aspartokinase, homoserine dehydrogenase, and threonine deaminase in a variety of microorganisms reveals that several distinct overall control patterns can be identified. In certain bacteria concerted action of two end products controls the early enzymes; in others, the existence of multiple forms of the same enzyme, each regulated by one metabolite, appears to be the principal mode of control. Compensatory effects of more than one modifier of opposing influence as well as the dual regulatory role of a single end product for two interconnecting pathways underline other alternative control characteristics. Each pattern appears adequate for a normal flow of metabolites from aspartate to various amino acid end products, and each scheme is peculiarly suited to the physiology and metabolism of the organism. The examples show that common enzymatic steps and similar control patterns do not necessarily reflect evolutionary affinities; diversity in the control mechanisms also a priori does not indicate independent phylogenetic relationships.

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35. The term compensatory feedback control used here depicts the phenomenon more accurately than the functional description such as specific reversal of feedback inhibition suggested in earlier publications (*6, 31*); this terminology was originally proposed by Sanwal and Maeba [*J. Biol. Chem.* **241**, 4557 (1966)] to describe the activation by various nucleotides of phosphoenolpyruvate carboxylase to replenish the concentration of oxaloacetate drained away from the tricarboxylic acid cycle for synthetic purposes.
36. Some selected examples of the ability of a second modifier to reverse the feedback inhibition control are given in (*32*).
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