Amino acids

MCB 113

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Industrial production of amino acids

Amino acid	World annual production (metric tons)	Methods	Use
L-Alanine	130	1,3c	Flavor enhancer
DL-Alanine	700	2	Flavor enhancer
L-Arginine	1,000	1, 3a	Infusion, cosmetics
L-Aspartate	4,000	1, 3c	Flavor enhancer, aspartame production
L-Asparagine	50	1, 2	Therapeutic
L-Cysteine	700	1	Bread additive, antioxidant
L-Glutamate	370,000	3a	Flavor enhancer
L-Glutamine	500	3a	Therapeutic
Glycine	6,000	2	Organic synthesis
L-Histidine	200	3a, 1	Therapeutic
L-Isoleucine	150	3a	Infusions
L-Leucine	150	1, 3a	Infusions
L-Lysine	70,000	3a, 3c	Feed additive, infusions
DL-Methionine	70,000	2	Feed additive
L-Methionine	150	3c	Therapeutic
L-Ornithine	50	3a, 3c	Therapeutic
L-Phenylalanine	3,000	3a, 3c	Infusions, therapeutic, aspartame production
L-Proline	100	3a	Infusions
L-Serine	50	3a, 3c	Cosmetics
L-Threonine	160	3a	Feed additive
L-Tryptophan	200	3a, 3c	Infusions, therapy
L-Tyrosine	100	1, 3c	Infusions, L-DOPA synthesis
L-Valine	150	3a, 3c	Infusions

Production methods: 1, hydrolysis of proteins; 2, chemical synthesis; 3a, direct fermentation; 3b, microbial transformation of precursors; 3c, use of enzymes or immobilized cells. Taken from Glazer and Nikaido.

Amino acids - some history

- Production of amino acids dates back to 1908
  - Japanese agricultural chemist K. Ikeda discovered that L-glutamate was responsible for taste of foods cooked with dried kelp
  - L-Glutamate was produced by acid hydrolysis of proteins --> expensive separation of other aa's
- In 1957, scientists at Kyowa Hakko discovered a soil bacterium that excreted large amounts of L-glutamate.
  - Similar bacteria have been discovered since that time.
- Annual amino acid production had a total value of US$1.9 billion by 1979.

Amino acid use

- Most use of amino acids is associated with food.
  - Flavor enhancers (primarily L-glutamate)
  - Feed additives (lysine, methionine, tryptophan, leucine, isoleucine, valine, phenylalanine, threonine, and arginine are not synthesized by higher animals)
- Amino acids are also used for the production of other compounds
  - Aspartame, the sweetener, is produced from L-phenylalanine and L-aspartate.

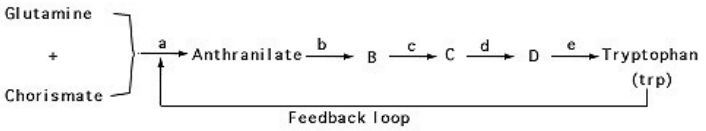
Fermentation

- Different from production of ethanol
  - Organism derives benefit from producing ethanol (dispose of NADH)
- Cells derive no benefit from excreting amino acids -- waste of energy and carbon
  - Amino acid production is usually effectively regulated
  - Most microbes do not want to waste amino acids
- Biosynthesis is regulated at two levels
  - Control of activity of pre-existing enzymes
  - Control of synthesis of new enzyme molecules

Enzyme activity control

- Allows for rapid down-regulation of biosynthesis
- Amino acid production is energy- and carbon-expensive
  - Reduction of synthesis when amino acid is present in the environment allows the cells to use the energy and carbon for faster growth
- Usually achieved through feedback inhibition
  - Excess end product inhibits the activity of the first enzyme of the biosynthetic pathway

Feedback inhibition

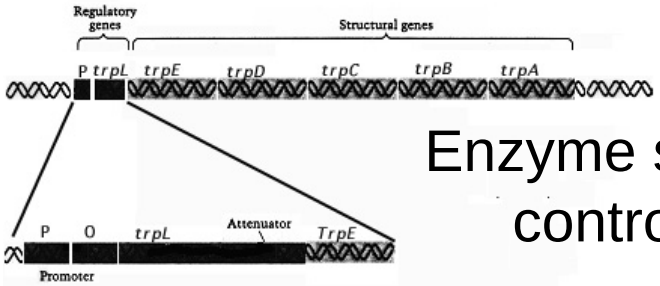


- The pathway of tryptophan biosynthesis in *E. coli*.
- The pathway is regulated by the process of feedback inhibition.
- Tryptophan (trp), the end product of the pathway, is the effector molecule that binds to the allosteric site of Enzyme a, the first enzyme in the pathway.
- When trp is bound to the enzyme the catalytic (active) site of Enzyme a is altered so that it is unable to react with its subtrates and the synthesis of anthranilate is inhibited.

Taken from http://textbookofbacteriology.net/regulation.html

Enzyme synthesis control

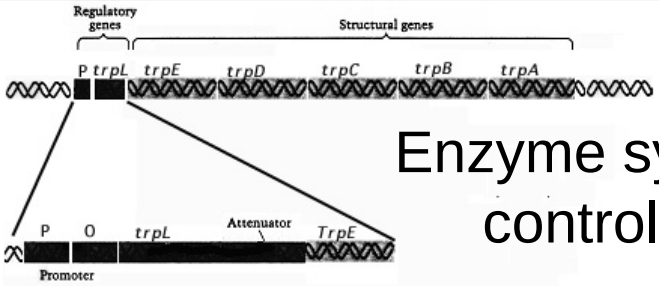
- Enzyme synthesis can be regulated by one of two mechanisms
  - Repression
    - amino acid end product of the pathway binds to a specific repressor protein
    - Repressor protein-amino acid complex regulate transcription
  - Attenuation
    - Controls the frequency of RNA chain termination during transcription



Enzyme synthesis control: Trp

- Genetic organization of the Trp operon and its control elements
- R = Regulatory gene that encodes for the trp Repressor protein that is concerned with regulating the synthesis of the 5 gene products. An active repressor binds to a specific nucleotide sequence in the operator region and thereby blocks binding of RNAp to the promoter to initiate transcription.
- O = Operator specific nucleotide sequence on DNA to which an active Repressor binds.
- P = Promoter specific nucleotide sequence on DNA to which RNA polymerase binds to initiate transcription. If the repressor protein binds to the operator, RNAp is prevented from binding with the promoter and initiating transcription. Therefore, none of the enzymes concerned with tryptophan biosynthesis are synthesized.

Taken from http://textbookofbacteriology.net/regulation.html

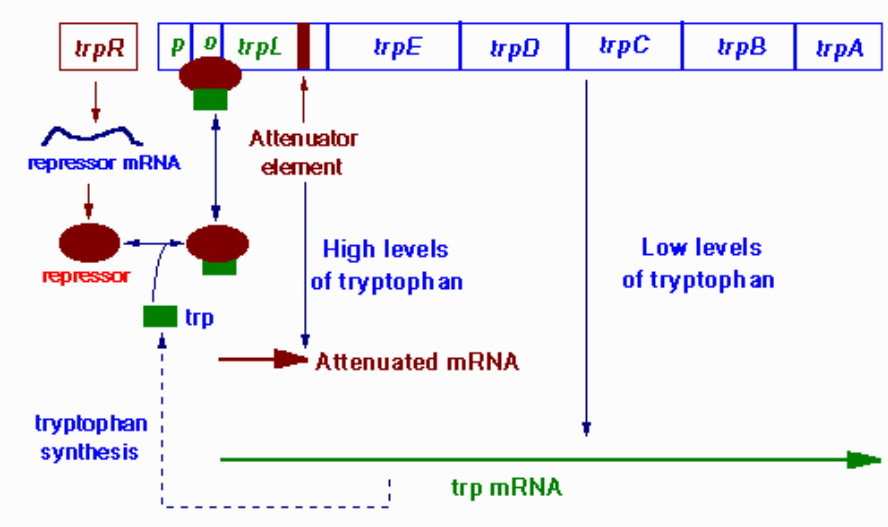


Enzyme synthesis control: Trp

- A = Attenuator DNA sequence which lies between the operator and the structural genes for trp biosynthesis. The attenuator is a barrier that RNA polymerase must traverse if it is to transcribe the genes for tryptophan biosynthesis. In the presence of trp, most RNAp molecules fall off the DNA before transcribing the trp genes. In the absence of trp, RNAp is able to traverse the attenuator region to successfully transcribe the trp genes.
- Trp A, B, C, D, E = Trp Genes structural genes for enzymes involved in tryptophan biosynthesis.
- Trp = tryptophan end product of the tryptophan biosynthetic pathway. When combined with the repressor protein the Repressor is active. Trp is called a corepressor.

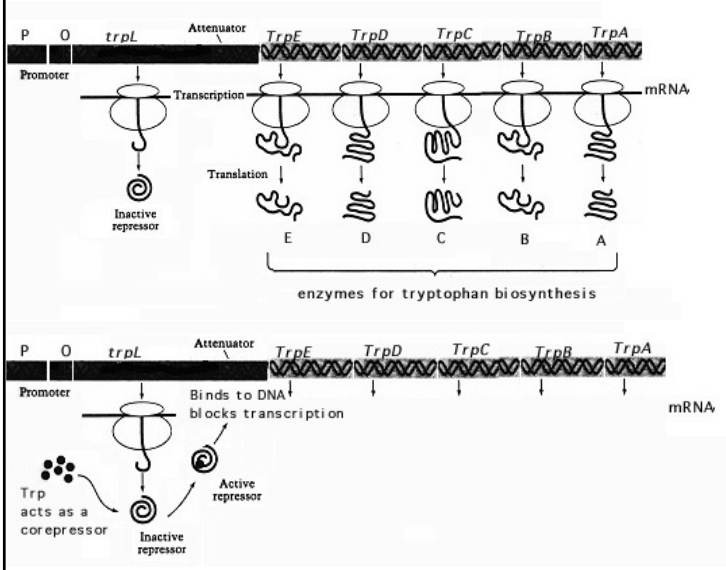
Taken from http://textbookofbacteriology.net/regulation.html

Structure of the *trp* operon



http://www.indstate.edu/thcme/mwking/gene-regulation.html

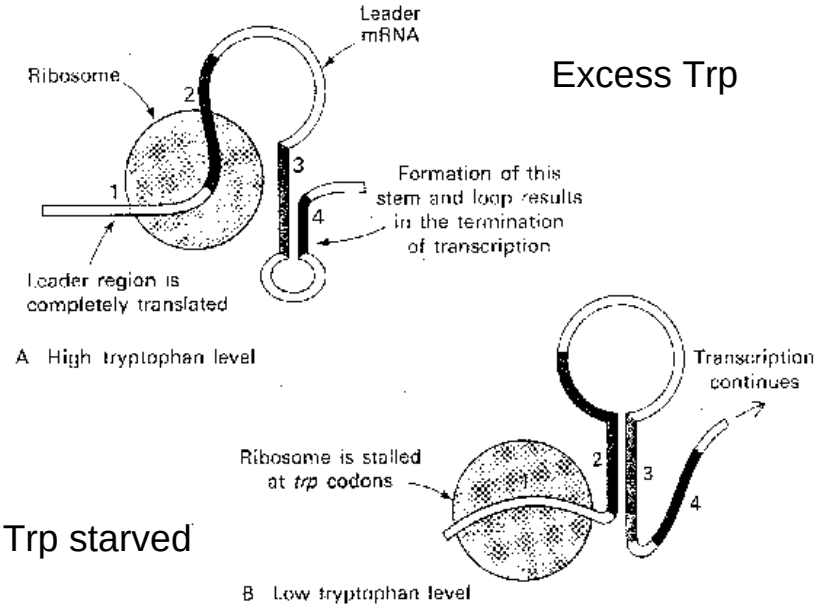
Regulation of Trp operon



No Trp

Trp Present

Attenuation in the Trp operon



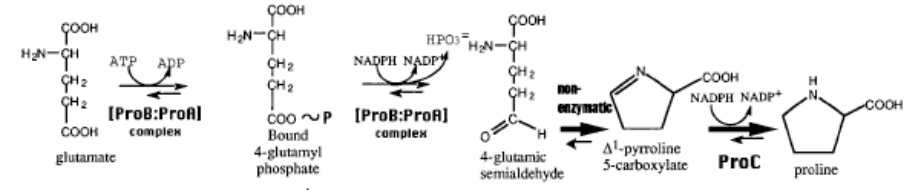
Auxotrophic mutants

- Cells are blocked for synthesis of a particular amino acid
  - Obtained in various ways
- Intermediates in the amino acid pathway are overproduced
  - L-Ornithine and L-citrulline are intermediates in the L-arginine biosynthetic pathway
  - L-ornithine-producing cells are mutants of the arginine biosynthetic pathway
- Drawback
  - One or more amino acids will not be produced by these mutants
  - The amino acid that is lacking must be added to the medium to allow the cells to grow

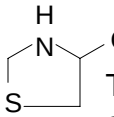
Regulatory mutants of unbranched pathways

- Regulatory mutants can be isolated by using an amino acid analog
  - The amino acid analog mimics the way the amino acid naturally regulates its biosynthesis but cannot be incorporated into protein
  - The only way the cell can grow is to obtain a mutant of the regulated step so that the cells can produce the amino acid
- In *E. coli*, amino acid biosynthesis is regulated at several steps so this method is not always effective.
  - The method is more effective for soil bacteria (e.g., *Serratia marcescens*) where there is less regulation.

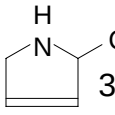
Regulatory mutants of unbranched pathways



- Proline mutants
  - Generated using the proline analogs thiazolidine-4-carboxylic acid and 3,4-dehydroproline



Thiazolidine-4-carboxylic acid



3,4-Dehydroproline

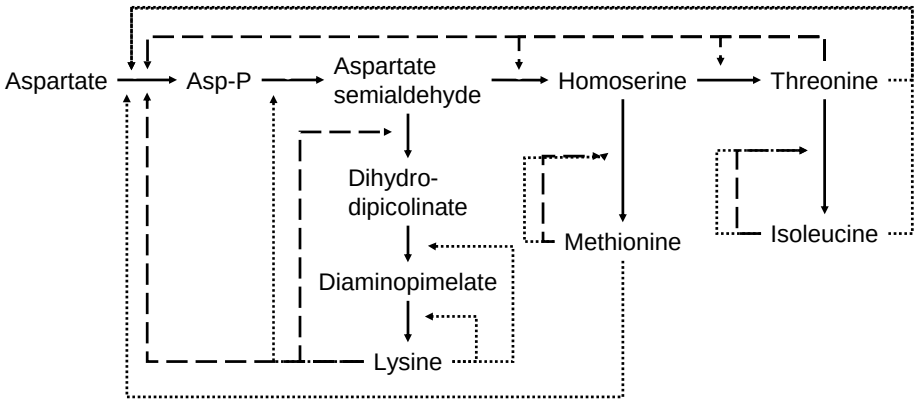
Regulatory mutants of unbranched pathways

- Maximizing proline production required two additional mutants
  - A mutation in the proline degradation pathway
  - Proline is used as an osmoprotectant
    - Forcing cells to grow in medium with high salt lead to additional mutants
- More recent technology uses metabolic engineering to increase flux through the pathway

Regulatory mutants of branched pathways

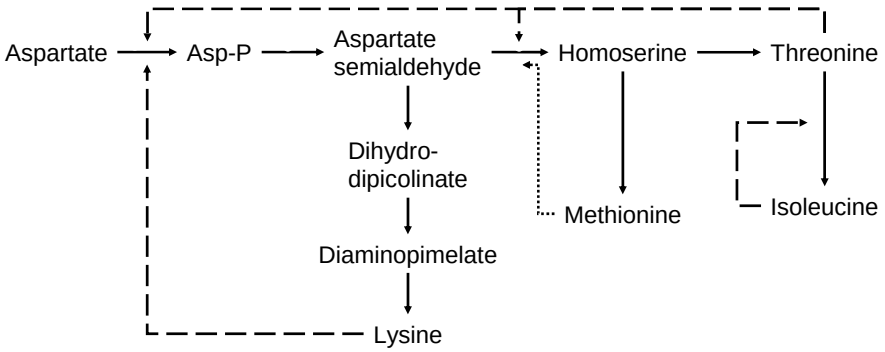
- Examples
  - Aspartate family of amino acids (lysine, methionine, threonine, isoleucine)
  - Pyruvate family (tryptophan, phenylalanine, tyrosine)
- Regulation is complicated
  - Generation of mutants is very difficult due to the complex regulation
  - Some organisms (e.g., *Brevibacterium flavum*, *Corynebacterium glutamicum*) have less complicated regulation

Regulation of the aspartate family of amino acids in *E. coli*



Solid lines: reactions
Dashed lines: regulation of enzyme activity by feedback inhibition
Dotted lines: regulation of gene expression by repression or attenuation

Regulation of the aspartate family of amino acids in *Brevibacterium flavum*



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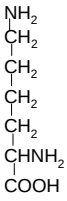
Production by auxotrophic mutants

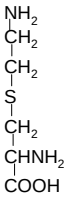
- The simple regulation of aspartate amino acids in *B. flavum* makes it simple to overproduce a particular amino acid
  - Delete genes for other branches in the pathway
  - Deletion of the branches leading to methione and to threonine and isoleucine lead to a 34-gram/L yield of lysine.
  - Methionine, threonine,and isoleucine must be continuously fed to the cells
    - Large excesses of these amino acids inhibits production.

Production by regulatory mutants

- Regulatory mutants of *Brevibacterium flavum* were obtained using the lysine analog S-aminoethylcysteine (AEC)
- Analog inhibits activity of aspartate kinase and growth of the wild-type bacteria
- AEC-resistant mutants have alteration in their aspartate kinase



Lysine

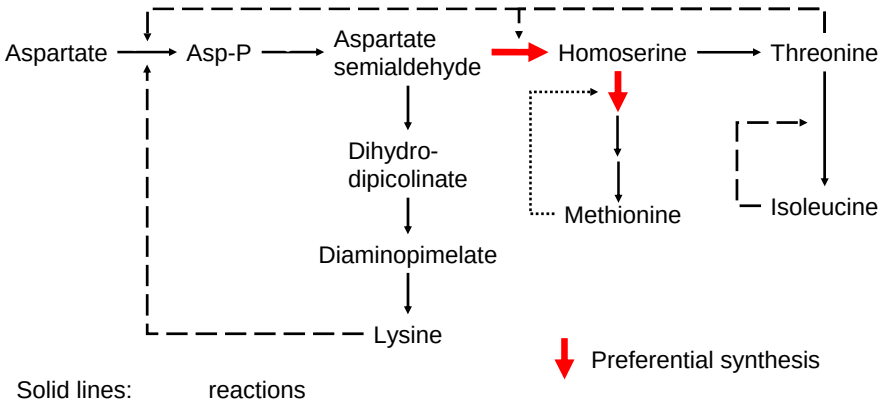


S-aminoethyl-cysteine

Preferential synthesis

- Simplest way to regulate a branched pathway is to have very unbalanced activities at the branchpoint
- Activities of enzymes after the branchpoint must be regulated
  - Activation of the weaker enzyme
  - Inhibition/repression of the stronger enzyme
- In *B. flavum*, the activity of the first enzyme in the homoserine branch is 15 times higher than that of the first enzyme of the lysine branch.
  - Same thing for methionine branch versus threonine/isoleucine branch
  - Product of weaker branch regulates the first enzyme in the pathway

Preferential synthesis in the aspartate family of amino acids in *Brevibacterium flavum*



Solid lines: reactions
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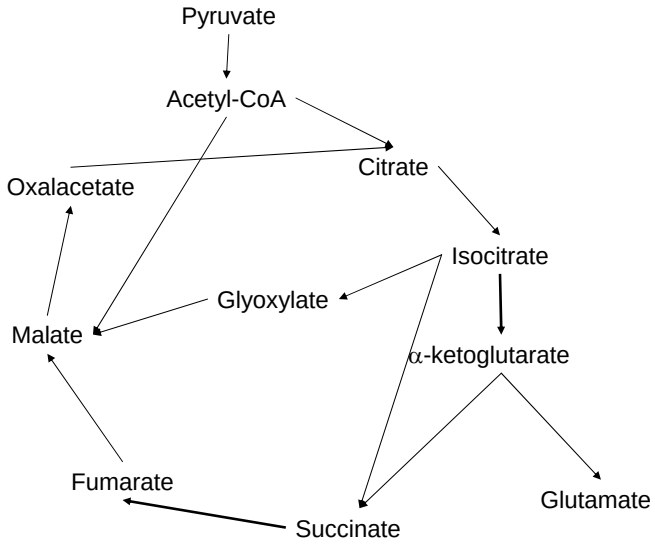
Fermentation with wild-type strains

- Membrane permeability
  - Important to get the amino acid out of the cell
  - Glutamate overproducers (*Brevibacterium-Corynebacterium*) secrete glutamate
    - Overproducers require biotin for growth
    - Adding excess biotin to the medium limits glutamate secretion
    - Biotin is needed for synthesis of fatty acids
    - Insufficiency of biotin leads to insufficiency of fatty acid synthesis and increased membrane permeability

TCA cycle and glyoxylate bypass



Fermentation with wild-type strains

- Functionally truncated citric acid cycle
  - Many *Brevibacterium-Corynebacterium* strains excrete large amounts of glutamate into the medium --> 100 g/L
  - Must have alterations in the TCA cycle to get such high production levels
    - The *Brevibacterium-Corynebacterium* group has an α-ketoglutarate dehydrogenase that is extremely labile
    - α-ketoglutarate accumulates and leads to glutamate synthesis
    - These microbes use the glyoxylate shunt, which is regulated by oxygen
      - Glutamate is produced maximally at low oxygen concentrations

Fermentation with wild-type strains

- Membrane permeability
- Functionally truncated citric acid cycle
- Regulation of enzymes in glutamate synthesis

Amino acid fermentation and recombinant DNA technology

- It is often not essential, or not possible, to use recombinant DNA technology for amino acid biosynthesis
  - Amino acid biosynthetic machinery already exists in cells
  - Regulatory mutants of amino acid biosynthetic enzymes have high activity and do not need to be overexpressed
  - All enzymes in the pathway must be balanced to get appropriate flux through the pathway
  - In many cases, yield is affected more by the availability of starting materials

Case study: L-tryptophan production by rDNA

- Emergence in 1980's of a disease called eosinophilia-myalgia syndrome
  - Two dozen deaths and severe illness in hundreds
  - Found in people who consumed large quantities of L-tryptophan from batched made by Showa Denko in Japan
- L-tryptophan produced in recombinant *Bacillus amyloliquefaciens*
  - Trace amounts of tryptophan 1,1'-ethylidene-bis[tryptophan] and ditryptophan 3-anilino-L-alanine were found in batches
  - Could have caused the disease
  - Although several other things changed in the process (filtration steps, activated charcoal), outbreak coincided with use of recombinant microbe.

Amino acid production with enzymes

- Some enzymes are produced using simple enzymatic reactions (outside the cell)
  - Amino acids can be produced in high concentrations
  - Purification is simpler since there are fewer components
  - Rate of production is higher than in fermentations
- Example: Production of aspartate using fumaric acid and NH3
  - Aspartase does conversion
  - L-Aspartate is used for synthesis of Aspartame, the sweetner

L-aspartic acid --> Fumaric acid + NH_3

Amino acid production with enzymes

- Enzymes must be immobilized to make the process economical
  - Allows for enzyme recovery
  - Simplifies purification