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Multifunctional Enzymes and Evolution of Biosynthetic Pathways: Retro-Evolution by Jumps

Siddhartha Roy*
Department of Biophysics, Bose Institute, Calcutta, India

ABSTRACT **A likely scenario of evolution of biosynthetic pathways is believed to have occurred by retro-evolution through recruitment of existing enzymes rather than generation of de novo classes. It had been proposed that such retro-evolution occurred in steps as a response to depletion of an essential metabolite and availability of another related substance in the environment. In this article, I argue that because of instability of many such extant intermediates, it is unlikely that retro-evolution had occurred in steps. I further propose that such evolution in many cases has taken place by jumps, i.e., by recruitment of a multifunctional enzyme capable of catalyzing several steps at a time, albeit inefficiently. I further speculate that in some cases one primordial multienzyme may have catalyzed the whole sequence of reaction of a biosynthetic pathway, i.e., the pathway may have evolved by a single leap. Gene duplications and further evolution to more efficient enzymes led to extant pathways. Such a mechanism predicts that some or all enzymes of a pathway must have descended from a common ancestor. Sequence and structural homologies among extant enzymes of a biosynthetic pathway have been examined. Proteins 1999;37:303–309.** © 1999 Wiley-Liss, Inc.

Key words: pathway; biosynthesis; evolution; multifunctional; enzyme

INTRODUCTION

Evolution of cells dependent on prebiotic soup for essential components to cells that are fully capable of synthesizing such essential components from compounds that are widely available in nature, must have been a very important step toward further evolution of life. One biochemical feature that distinguishes the latter type of cell from its putative early precursors is the presence of metabolic pathways. However, how such metabolic pathways evolved is still not well understood. The mechanism of evolution of enzymes of a metabolic pathway poses a difficult problem. Many enzymes in metabolic pathways catalyze transformations between two chemical species that are not used in any other functions of the cell. The utility of such enzymes to the cell is only as a part of that specific metabolic pathway as a whole. Thus, it is unlikely that the component enzymes of a metabolic pathway evolved separately without the end product in place and that could be used by the cell.

A number of investigators have speculated about the mechanism of such evolution, particularly that of the biosynthetic pathways. Notable among them is Horowitz.[1,2] His hypothesis can be described as retro-evolution in steps. Initially, the cell used certain useful metabolites directly from the environment. As one of the metabolites became scarce, the cell developed an enzyme that converted another related compound, available in the environment, to the scarce metabolite. The whole biosynthetic pathway then developed by repetition of this procedure. The scarcity of a metabolite did not have to be drastic, and the requirement of the new enzyme did not have to be very efficient at the initial stage. A relatively small drop in the concentration of an essential metabolite may have been sufficient to alter the growth rate of a cell so that a relatively inefficient enzyme would confer enough of an advantage. As a result, in several generations the latter species would overtake the former one.

Zuckerkandl[3] argued that most protein classes originated before the major elements of protein synthesis apparatus were fully evolved. Assuming that de novo generation of new classes, under such selection pressures, is unlikely—the central problem in the evolution of a metabolic pathway becomes enzyme recruitment, i.e., how an existing enzyme could be recruited to perform a new job. Because of the complex nature of metabolic pathways and evolution of different pathways under different environmental pressures, along with different genetic resources available to the evolving species at the time, it is unlikely that a single mechanism of recruitment was followed all the time. Ycas,[4] Waley,[5] and Jensen[6] argued that primitive cells contained a relatively small number of enzymes, catalyzing a class of reaction with broad substrate specificity. These broad-specificity enzymes then may be recruited for a new metabolic pathway, followed by further evolution toward more specific and efficient catalysts.

Acknowledging that this type of recruitment probably occurred frequently,[7] I suggest an alternative proposal based on the multifunctional nature of many enzymes. This hypothesis, which is not necessarily mutually exclusive with that of the currently favored concept of enzyme recruitment, is described below along with some structural evidences and underlying chemical logic.

*Correspondence to: Siddhartha Roy, Department of Biophysics, Bose Institute, P-1/12 C.I.T. Scheme VII M, Calcutta, 700 054, India. E-mail: siddarth@boseinst.ernet.in and sandmroy@cal2.vsnl.net.in
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The Hypothesis

Sequence of reactions that form a metabolic pathway is not always the simplest nor does it conform to what would be expected from the knowledge of organic reaction mechanisms. They evolved to their present state under constraints that are not fully understood. Retro-evolution by steps offers a rationale for the extant sequence of reactions. However, one of the problems in retro-evolution by steps is that some intermediates in biosynthetic pathways are unstable and, therefore, are unlikely to accumulate in sufficient concentration to permit stepwise retro-evolution. A well-documented case is that of 5-phospho-β-D-ribosylamine, a key intermediate in de novo purine biosynthesis. In free solution, it has a half-life of only 5 s, making it impossible to accumulate.[8] This leads me to suggest that only a few of the extant intermediate compounds were actually present in sufficient quantity, and one multifunctional enzyme may have been recruited to convert an intermediate to the final product. We may call this retro-evolution by jumps.

Obviously, how these multifunctional enzymes came into being is the critical question. I propose that in the primitive cells the existing enzymes possessed other potentially weaker catalytic activities. Albery and Knowles[9] suggested that enzymes evolved through three distinct stages: uniform binding of substrates, differential binding, and catalysis of elementary reaction steps, in that order. The potential catalytic activity, referred to above, is possibly the capability of substrate binding, which one can foresee being converted easily to a relatively inefficient enzyme using only uniform substrate binding—the suggested first step in the evolutionary pathway to an efficient enzyme.[9] The rate enhancement that could be obtained from uniform binding of substrates is significant. Page estimates that such rate enhancement may be as much as 10^{11} but more likely to be in the order of 10^{8} for bimolecular reactions.[10] I suspect that these additional substrate-binding capabilities before recruitment did not exist fortuitously but were selected. In primitive cells, the presence of proteins capable of binding essential metabolites may have conferred survival advantage by increasing the concentrations of the essential metabolites within the cell.

It is now known that enzymes catalyzing diverse classes of reaction may have a common fold. A classic example is that of closed α/β barrel fold. Enzymes catalyzing oxidation, phosphorylation, and isomerization are known to belong to this fold.[11] Other folds that catalyze different classes of chemical reactions are also known. I propose that a protein that existed in the cell for the reason of catalyzing a vital reaction also possessed a capability of binding multiple metabolites. Under selection pressure of a fast depleting essential metabolite, this protein may be recruited with a small number of mutations as a multifunctional enzyme capable of catalyzing multistep conversion of an available intermediate to the depleting metabolite. This is not surprising, because many modern day proteins are known to have the capability of catalyzing more than one reaction. A well-documented example is that of carbamoyl phosphate synthetase.[12] This deceptively simple reaction was recently shown to be composed of four distinct reactions having three unstable intermediates and catalyzed by a single enzyme at four distinct active sites. Some transferred groups actually travel 100 Å within the enzyme during a catalytic cycle! Another well-documented example is that of dehydrquinate synthase, which conducts five consecutive reactions of oxidation, β-elimination, reduction, hydrolysis, and intramolecular aldol condensation in the same active site.[13] Some known examples of some bifunctional enzymes are summarized in Table I.

TABLE I. Bifunctional Enzymes in Biosynthetic Pathways

Enzymes	Biosynthetic pathway	Reference
Dehydroquinate synthase	Aromatic aminoacid biosynthesis	Carpenter et al.[13]
Carbamoyl phosphate synthetase	Pyrimidine and argininge biosynthetsis	Raushel et al.[12]
β-hydroxydecanoyl thiol ester dehydrase	Unsaturated fatty acid biosynthesis	Leesong et al.[32]
L-histidinol dehydrogenase	Histidine biosynthesis	Teng et al.[33]
Methylene folate dehydrogenase/cyclohydrolase	Folate biosynthesis	Allaire et al.[34]
FAD synthetase	FAD biosynthesis	Efimov et al.[35]

In general, most biosynthetic pathways use a limited number of chemical components to arrive at the final products and often use them more than once during the whole sequence of reactions. As an example, de novo purine biosynthesis consists of 13 enzyme-catalyzed steps but uses only six distinct chemical species, including adenosine triphosphate.[14] Thus, a capability of binding a relatively small number of substrates can provide the potential ability to be converted to a multifunctional enzyme. Further evolution toward more efficient catalysts may have taken place by successive gene duplications, loss of all, but one, catalytic functions, and enhancement of that remaining catalytic power.

RESULTS AND DISCUSSION

One of the critical tests of the retro-evolution by jumps model is the existence of sequence or structural homology for enzymes that catalyze consecutive steps. Because it is likely that the extant enzymes diverged from the common multifunctional ancestor in the distant past, a demonstrable sequence homology may not always be expected. However, I expect to see three-dimensional structural homology between some enzymes, catalyzing contiguous reactions. In the following section, biosynthetic pathways were analyzed for supportive evidences.

Known Homology Between Enzymes of Biosynthetic Pathways and Existence of Bifunctional Enzymes

Structural homology between enzymes of a metabolic pathway has been noted before, although it has been

TABLE II. Homology Between Enzymes of a Biosynthetic Pathway

Enzyme	Biosynthetic pathway	Method	Reference
N1-((5′-phosphoribosyl)-formimino)-5-aminoimidazole-4-carboxamide ribonucleotide isomerase (hisA) and imidazole glycerol phosphate synthase (hisF)	Histidine biosynthesis	Sequence comparison	Thoma et al.[36] Bork et al.[37]
ThiE and ThiG proteins of *E. coli*	Thiamin biosynthesis	Sequence comparison	Bork et al.[37]
Cystathionine γ-synthase and cystathionin β-lyase	Methionine biosynthesis	Sequence and structure comparison	Clausen et al.[19] Belfaiza et al.[18]
Aspartokinase and homoserine kinase	Threonine biosynthesis	Immunological cross-reactivity	Truffa-Bachi[38]
Phosphoribosyl anthranilate isomerase, indole glycerol phosphate synthase, and tryptophan synthase α-subunit	Tryptophan biosynthesis	Structure comparison	Branden and Tooze[11]
D-diaminopimelate dehydrogenase and dihydropicolinate reductase	Lysine biosynthesis	Structure comparison	Scapin et al.[21]

attributed to convergent evolution. It has been known that some, if not all, enzymes of the glycolytic pathway are structurally similar.[15] There are three pairs of enzymes that catalyze consecutive reactions and also have identical topologies: aldolase-triosephosphate isomerase, glyceraldehyde phosphate dehydrogenase-phosphoglycerate kinase, and enolase-pyruvate kinase. Enolase and pyruvate kinase may originate from a common ancestor.[16] Recently, it was suggested that classical Embden-Meyerhof-Parnas pathway of glycolysis was originally an anabolic pathway and only later adapted to its current catabolic role[17]; thus, it is suggestive that this extant catabolic pathway originally evolved as a biosynthetic one with three ancestral multifunctional enzymes: aldolase-triosephosphate isomerase, glyceraldehyde phosphate dehydrogenase-phosphoglycerate kinase, and enolase-pyruvate kinase. In addition, many enzymes are known to catalyze more than one consecutive reaction in a pathway. Such extant enzymes offer a clue toward possible multistep catalysis by a primordial enzyme. I have not included any known example of multifunctional enzymes that are a mere fusion of two or more enzymes.

Biosynthesis of methionine

Biosynthesis of methionine occurs from homoserine and consists of four enzyme-catalyzed steps. The second and third steps are catalyzed by cystathionine-γ-synthase and cystathionine-β-lyase. It has been shown that these two enzymes have strong sequence homology.[18] Recent three-dimensional structure determinations show that the folds of these enzymes are also very similar.[19]

Diaminopimelic pathway of bacteria

The diaminopimelic acid pathway of lysine biosynthesis in bacteria and higher plants vary, depending on the organism. All organisms having diaminopimelic acid pathway have the first four steps, from aspartate to L-Δ^1-piperidine-2,6-dicarboxylate, in common. They are catalyzed by four enzymes: aspartate kinase, aspartate semialdehyde dehydrogenase, dihydropicolinate synthase, and dihydrodipicolinate reductase. The pathway can then take either of the following three routes to meso-diaminopimelate: (a) via succinylation involving four steps (b) via acetylation involving four steps and (c) catalyzed by a single-enzyme D-diaminopimelate dehydrogenase.[20]

The last pathway, meso-diaminopimelic acid, which is used in cell wall biosynthesis, is likely to be a key intermediate and widely available. The key enzyme in this pathway is D-diaminopimelate dehydrogenase, for which the crystal structure has been determined. This protein has two domains: the N-terminal one is Rossman fold, and the C-terminal one is the same fold and architecture but different topology (luciferase, domain 4). The crystal structure of the preceding enzyme in the pathway, dihydropicolinate reductase, has also been determined. It also has two domains: the N-terminal domain is Rossman fold, and the C-terminal domain has two-layer sandwich α-βfold. In fact, it has been suggested that they evolved from a common ancestral multifunctional protein having both activities.[21]

Aromatic amino acid biosynthesis

Biosynthesis of phenylalanine, tyrosine, and tryptophan consists of a common seven-step pathway to chorismic acid from erythrose-4-phosphate and phosphoenol pyruvate. The individual amino acids then branch off from this point.[14] Let us follow the branch that leads to tryptophan from chorismic acid. Because chorismic acid stands at the fork of a metabolic pathway and it is abundantly distributed in nature, it is likely that this was an abundant intermediate, to which retro-evolution might have taken place. The branch that leads to tryptophan is composed of four steps catalyzed by the following enzymes: anthranilate synthase, anthranilate phosophoribosyl transferase-indole glyceroyl phosphate (PRA-IGP), and tryptophan synthase. PRA-IGP is a bifunctional enzyme and the two reactions are catalyzed independently on two different domains of the enzyme. The α-subunit of tryptophan synthase catalyzes the first partial reaction of tryptophan synthesis, i.e., hydrolysis of indole-3-glyceroyl phosphate to indole and glyceraldehyde-3-phosphate. Crystal structures of PRA, IGP, and the α-subunit of TS show that they

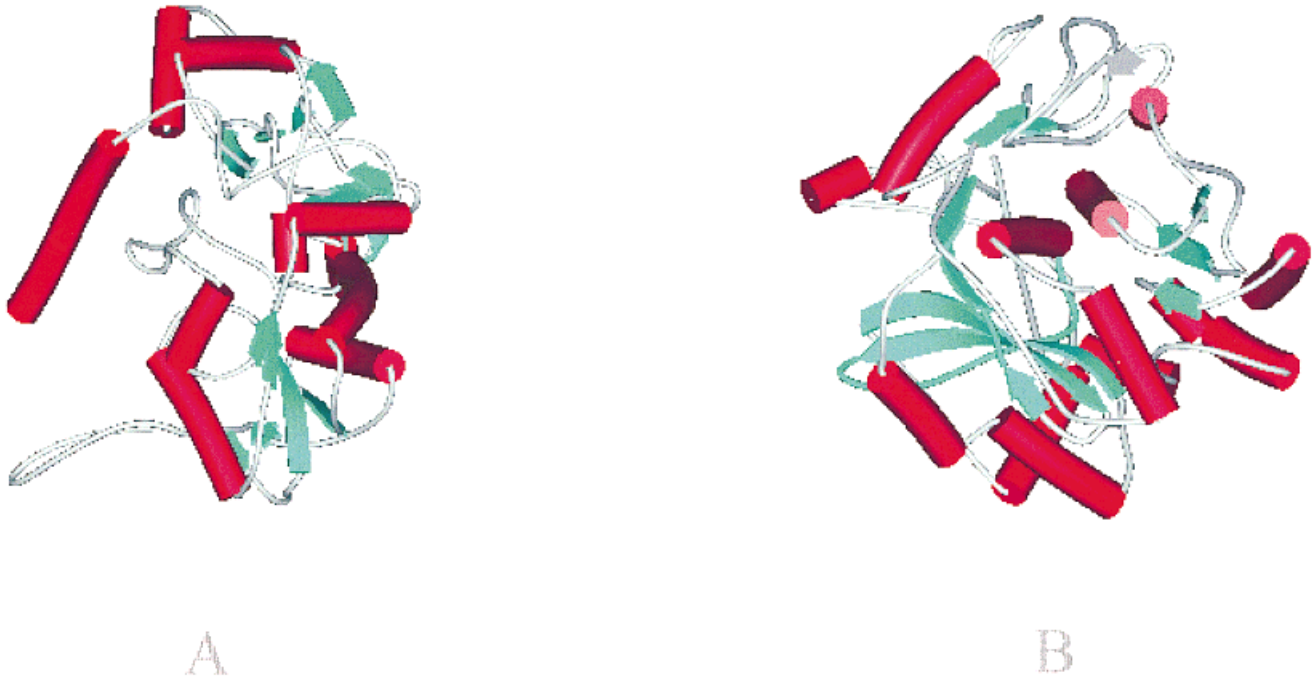

Fig. 1. **A:** Cartoon drawing of glutamine phosphoribosyl amido transferase. **B:** Cartoon drawing of glycinamide ribonucleotide synthetase. The N-terminal domain of glutamine phosphoribosyl amidotransferase, which binds glutamine, as well as a small domain (120–190) of glycinamide ribonucleotide synthetase has been deleted from the figures for clarity.

Fig. 2. **A:** Cartoon drawing of class 3 aldehyde dehydrogenase (rat; PDB:1ad3). **B:** L-2-hydroxyisocaproate dehydrogenase (lactobacillus confusus; PDB:1hyh).

TABLE III. Domains of Enzymes of Purine Biosynthetic Pathway

Enzymes	Domains	Cath/reported classification
Glutamine phosphoribosylpyrophosphate amido transferase	Domain 1 (Residues 1–259, 380–390, 425–458)	Class: αβ; Architecture 4-layer sandwich
	Domain 2 (Residues 260–305, 328–379, 391–424)	Class: αβ; Architecture 3-layer αβα sandwich
Glycinamide ribonucleotide synthetase	Domain N (Other A, B, C)	Class: αβ; Architecture 3-layer αβα sandwich
Phosphoribosylglycinamide formyltrasferase	Single domain	Class: αβ; Architecture 3-layer αβα sandwich
SAICR synthase	Domain 2	Class: αβ; Architecture 3-layer αβα sandwich

TABLE IV. Predicted Folds of Enzymes of Proline Biosynthesis

Enzyme	Predicted fold	*Z*	RMSD	Alignment score
Pyrroline-5-carboxylate reductase (ProC)	L-2-hydroxyisocaproate dehydrogenase (1hyh)	6.96	2.9 Å	3.8
γ-glutamyl phosphate reductase	Class 3 aldehyde dehydrogenase (1AD3)	9.47		

The folds were predicted by the method of Fischer and Eisenberg[26] using the UCLA-DOE fold recognition server. The structural alignments were conducted by using the DALI server for three-dimensional protein structure and database searches.[39] A *z* score >5 is considered significant, and an alignment score >2 is considered significant.

are α/β barrels.[11] However, two of the enzymes in the main pathway, shikimate kinase and dehydroquinate synthase, as well as chorismate mutase, the first enzyme in the phenylalanine and tyrosine biosynthesis, have a different architecture.[13,22,23] This leads me to suggest that phenylalanine and tyrosine branches were established separately, and indole and chorismic acid were the two abundant intermediates during retro-evolution. In this view, retro-evolution of tryptophan biosynthesis occurred in two steps: (a) recruitment of tryptophan synthase β-subunit first to synthesize tryptophan from two commonly available metabolite serine and indole (b) recruitment of α-subunit of tryptophan synthase-IGP-PRA-anthranilate synthase multifunctional enzyme to synthesize indole from chorismic acid.

Some biosynthetic enzymes having detectable sequence or structural homology are listed in Table II.

Structural Homologies Detected by Fold Comparison

De novo purine biosynthesis

As mentioned above, 5-phospho-β-D-ribosylamine is a very unstable compound and unlikely to have accumulated in sufficient quantities under aqueous conditions. Thus, the two enzymes that precede and succeed this compound in the pathway likely originated from a single multifunctional enzyme. Both of these extant enzymes, glutamine phosphoribosyl amido transferase and glycinamide ribonucleotide synthetase, have been crystallized and the structures determined by X-ray diffraction.[24,25] Figure 1 shows the ribbon diagram of both the enzymes. The N-terminal domain of glutamine phosphoribosyl amidotransferase, which binds glutamine, as well as a small domain (120–190) of glycinamide ribonucleotide synthetase has been deleted from the figures for clarity. Summary of class, architecture, topology, and homologous superfamily (CATH) classification of different domains of the four enzymes of purine biosynthetic pathway are shown in Table III. Clearly, one of the core domains of each enzyme has three-layer αβα sandwich architecture, suggesting a common lineage.

Proline biosynthesis

In the proline biosynthetic pathway of eubacteria, glutamic acid is first phosphorylated to γ-glutamyl phosphate, which then is reduced to glutamic acid semialdehyde. This is followed by a spontaneous ring closure to Δ^1-pyrroline-5-carboxylic acid, which is then reduced to proline in a nicotinamide-adenine dinucleotide phosphate-dependent reduction. γ-Glutamyl phosphate, a mixed anhydride, is very unstable in solution. The three enzymes that catalyze these reactions are γ-glutamyl kinase (ProB in *Escherichia coli*), γ-glutamyl phsophate reductase (ProA), and pyrroline-5-carboxylate reductase (ProC). No statistically significant sequence homology was found between the enzymes, although some homologous local alignments were found. Thus, I have attempted to model the folds of these enzymes by the method of Eisenberg and co-workers.[26] The method predicts that ProA fold is similar to class 3 aldehyde dehydrogenase (rat; PDB:1ad3) and ProC fold is similar to l-2-hydroxyisocaproate dehydrogenase (lactobacillus confusus; PDB:1hyh) with *z* scores well beyond the threshhold. ProB, on the other hand, did not align well with any known fold (*z* scores were all less than threshold). When three-dimensional structures of 1ad3 and 1hyh were aligned by using the algorithm of Holm and Sanders, a significant part showed good alignment (*z* value 3.8; threshold of 2.0 and rms deviation of 2.9 Å). Table IV summarizes these results, and Figure 2 shows the folds of l-2-hydroxyisocaproate dehydrogenase and class 3 aldehyde dehydrogenase.

Did Metabolic Pathways Evolve by Leaps?

I have presented some evidences and arguments that retro-evolution occurred not only by steps but also sometimes by jumps, i.e., by recruitment of one multifunctional enzyme that catalyzed more than one step in the pathway. Extending that logic, one can ask, is there any evidence that one ancestral enzyme might have catalyzed all the reactions of a biosynthetic pathway? The case of proline

biosynthesis presented above is instructive. We have already detected similarity between the enzymes catalyzing the last two steps of the pathway. Both the enzymes are predicted to have Rossman fold. Although little could be inferred about the structure of ProB, the enzyme catalyzing the first step, it is possible that being a kinase it may have a Rossman-like fold. In fact, secondary structure prediction shows significant alternate βα patterns (data not shown), particularly in the C-terminal half, thus arguing in favor of such a possibility.

In another case, it was shown that a significant part of the fold of the first two enzymes of the purine biosynthetic pathway is similar. The third enzyme of the pathway, phosphoribosyl glycinamide formyltransferase, also has the same fold.[27] In addition, SAICAR synthase (seventh enzyme in the pathway) also has a domain of similar fold.[28] Based on computer search Galperin and Koonin[29] predict a domain of a similar fold for another enzyme in the pathway, phosphoribosylaminoimidazole carboxylase. This may indicate that the whole pathway originated from a single ancestral multifunctional enzyme catalyzing all the reactions.

On the other hand, many examples where enzymes of a metabolic pathway have completely different folds are known, suggesting that their recruitment happened from different classes of proteins and separately. Pyrimidine biosynthetic pathway is an example of such a kind of recruitment. Two of the enzymes of the pathway, dihydroorotate dehydrogenase and orotate phosphoribosyl transferase, for which the crystal structures are known, belong to two different groups of architecture, TIM barrel and Rossman fold (CATH classification). In the lysine biosynthesis pathway, tetrahydrodipicolinate-n-succinly transferase, dihydrodipicolinate synthase, and dihydrodipicolinate reductase belong to different architecture or different classes altogether.

In conclusion, I suggest that biosynthetic pathways evolved by recruitment of existing enzymes as a response to depleting metabolites—in steps, in jumps, and perhaps sometimes even by leaps.

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