ARTICLE

From RNA to DNA, Why So Many Ribonucleotide Reductases?

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It is generally accepted that DNA appeared after RNA during the chemical evolution of life. To synthesize DNA, deoxyribonucleotides are required as building blocks. At present, these are formed from the corresponding ribonucleotides through the enzymatic action of ribonucleotide reductases. Three classes of enzymes are present in various organisms. There is little sequence similarity among the three classes of reductases. However, enzymic mechanisms and the allosteric behavior of the enzymes from various organisms are strongly conserved, suggesting that the enzymes might have evolved from a common ancestor, with the class III anaerobic *Escherichia coli* reductase as its closest relative.

The chemistry of life rests on the properties of self-replication and catalysis. In its present form it involves the interplay of three classes of macromolecules—proteins, DNA, and RNA—with a clear distribution of labor among them. Proteins catalyze the chemical reactions that maintain the function of cells and organisms; DNA transmits by self-replication the blueprint for protein catalysts from one generation to the next; and RNA provides the link between the two by making available the information present in DNA for the synthesis of proteins.

During early evolution a simpler chemistry provided a mechanism for both self-replication and catalysis. RNA itself is able to transfer information and also has catalytic ability. At first, catalysis appeared to be limited to the formation of phosphodiester bonds and similar reactions (*1*), providing a primitive mechanism for the replication of RNA. More recently, RNA-mediated catalysis was implied also in the formation of peptide bonds (*2*). During evolution RNA was probably the first class of macromolecules to provide the means for both self-replication and catalysis. Proteins and DNA entered the "RNA world" at a later stage.

A different argument in favor of the precedence of RNA rests on how DNA and RNA are synthesized at present. Both are made from smaller building blocks, deoxyribonucleotides for DNA and ribonucleotides for RNA. These differ only in their sugar moieties. In ribonucleotides the second carbon atom (C-2′) of ribose is linked to a hydroxyl, whereas in deoxyribonucleotides C-2′ of deoxyribose is linked to hydrogen (Fig. 1). In all living organisms deoxyribonucleotides are made from ribonucleotides by a group of enzymes called ribonucleotide reductases (*3*). Probably the ability to reduce ribonucleotides had to exist at a time when both RNA and protein existed and before the appearance of DNA.

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Ribonucleotide Reduction

The enzymatic reduction of a ribonucleotide occurs with retention of configuration by the replacement of the hydroxyl at C-2′ with a hydrogen derived from the solvent. The reductant in the enzymatic reaction (Fig. 1) is a dithiol functionality of a small protein (thioredoxin or glutaredoxin) (*4*). When we postulated the existence of this type of reaction in the early 1950s on the basis of isotopic experiments with labeled nucleosides in the rat (*5*), we met with much skepticism; textbooks of organic chemistry did not describe such reactions. The solution came later with the observation that the enzyme is a radical protein, with one tyrosine residue of the polypeptide chain serving as the organic radical (*6*). Similar reactions can now be carried out in the test tube, without enzymes, by the application of radical chemistry.

The protein radical generates a substrate radical (Fig. 2) through a mechanism formulated by Stubbe (*7*). Its key feature is the abstraction of a hydrogen atom from C-3′ of the ribose by the protein radical. This facilitates the leaving of the hydroxyl group from the neighboring C-2′, which is reduced subsequently by the dithiol functionality.

Three classes of ribonucleotide reductases are known (*8*), containing enzymes of different primary protein structures. All are proposed to use a protein radical for the abstraction of the 3′-hydrogen, even though different strategies are followed for that purpose. Class I enzymes occur in all higher organisms and in some prokaryotes, with the *E. coli* enzyme as the prototype. In this enzyme Sjöberg identified Tyr^{122} as the radical. Class II enzymes, present in many prokaryotes, use adenosyl cobalamin as the radical generator, whereas class III enzymes, present in anaerobically grown *E. coli*, use *S*-adenosylmethionine (AdoMet) for this purpose.

In my comparison of the three classes, I will concentrate on three aspects: protein structure, generation and involvement of the protein radical, and allosteric regulation.

Iron-Tyrosyl Radical Enzymes (Class I)

These enzymes use ribonucleoside diphosphates as substrates. The *E. coli* reductase is the best characterized (*9*). It has an $\alpha_2\beta_2$ protein structure (with α_2 usually referred to as R1 and β_2 as R2) with a total molecular mass of 258 kD (Fig. 3). Each R1-protomer (85.7 kD) contains one substrate binding site with redox-active thiols for the reduction of ribose, and two separate allosteric sites.

R2 contains the tyrosyl radical Tyr^{122} and within each polypeptide (43.4 kD) an oxygen-linked iron [Fe(III)] center. The x-ray structure (*10*) reveals that Tyr^{122} is situated deep inside the protein.

The iron centers of R2 function in the generation and maintenance of the tyrosyl radical. Two forms of R2 lack the tyrosyl radical: (i) apoR2 is formed by treatment of R2 with iron-chelating reagents, resulting in the simultaneous loss of iron and the radical (*11*), and (ii) metR2 arises from treatment of R2 with radical scavengers, such as hydroxyurea, that leave the Fe(III) center intact (*12*).

Active R2 can be reconstituted from both apoR2 and metR2. Reconstitution

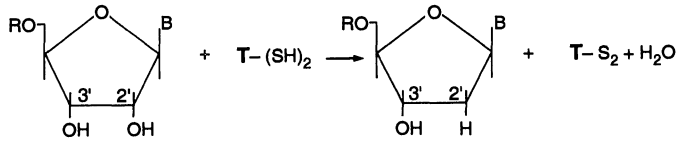

Fig. 1. Schematic representation of ribonucleotide to deoxyribonucleotide reduction. $T\text{-}(SH)_2$, thioredoxin or other small proteins containing redox-active thiols; R = PP_i (diphosphates; class I enzymes) or PPP_i (triphosphates; class III and most class II enzymes); B, base.

from apoR2 requires addition of Fe(II) in the presence of oxygen (*11*). The simultaneous oxidation of Fe(II) and Tyr^{122} reforms both the Fe(III) center and the tyrosyl radical. The introduction of the tyrosyl radical into metR2 is catalyzed by a complex enzyme system, with a flavin reductase occupying a pivotal role (*12*). Reduced flavins produced by this enzyme first reduce the Fe(III) center of metR2 to Fe(II). Oxygen subsequently oxidizes Fe(II) and Tyr^{122} to yield active R2. In both cases the formation of the tyrosyl radical requires the oxidation of an Fe(II) center in R2 to Fe(III) by molecular oxygen. This reaction is proposed to occur via a diferric peroxide intermediate with oxygen bound to the iron center (9). Experiments using rapid kinetics have led to the discovery of several intermediates in this reaction (*13*).

The tyrosyl radical was first believed to be directly involved in the abstraction of the hydrogen at C-3′ (Fig. 2). However, the x-ray structure indicates that Tyr^{122} is at a distance of 10 Å from the surface and therefore cannot interact directly with the catalytic site on R1. This suggests a more indirect function for Tyr^{122}

The participation of active thiols of R1 during catalysis (Fig. 2) was demonstrated by Thelander as early as 1974 (*14*). He found that 1 mol of R1 whose cysteines had been reduced chemically with dithiothreitol could, in the absence of any thioredoxin, reduce 3 mol of cytidine diphosphate (CDP) with the simultaneous oxidation of 6 mol of cysteine. This result suggested that R1 contains more than one redox-active dithiol per protomer.

From the amino acid sequence of R1, specific cysteine residues representing the active thiols were identified (*15, 16*). Five from a total of 11 cysteines are involved. Two cysteines near the COOH-terminus, Cys^{754} and Cys^{759}, appear to accept electrons from reduced thioredoxin and are proposed to relay them to a second pair, Cys^{225} and Cys^{462}, that are directly involved in the reduction of C-2′ (Fig. 2). The fifth cysteine, Cys^{439}, is suggested by Stubbe to form a thiyl radical and to abstract the hydrogen from C-3′. The tyrosyl radical on R2 is then viewed as a radical initiator that, by means of long-range electron transfer, generates the thiyl radical on Cys^{439}.

Allosteric control of class I enzymes. All pure ribonucleotide reductases reduce all four common ribonucleotides. Coordinated production of deoxyribonucleotides depends on a complicated allosteric control of their substrate specificity.

For the *E. coli* enzyme, as a representative of class I enzymes, extensive kinetic and binding experiments have led to a detailed understanding of its allosteric control that involves binding of nucleoside triphosphates (the substrate is diphosphates) to the R1 protein (*17*). Each protomer contains two different kinds of allosteric sites (Fig. 3).

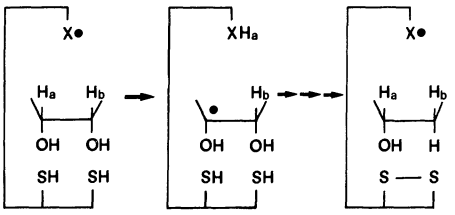

Fig. 2. Mechanism of ribonucleotide reduction proposed by Stubbe (*7*). The figure shows only the main features. The protein radical (X•, probably thiyl) abstracts a hydrogen atom from C-3′. This facilitates the leaving of the hydroxyl group at C-2′, which is reduced by thiols of the enzyme. The identical hydrogen atom abstracted by the protein radical is returned to C-3′.

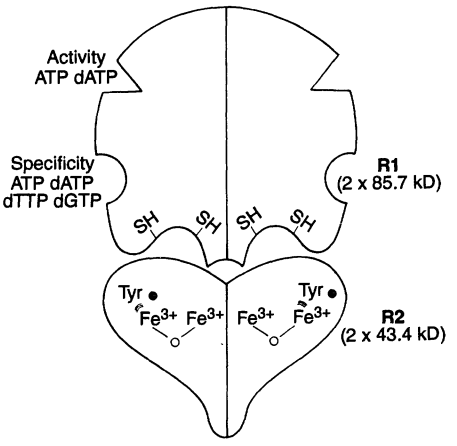

Fig. 3. Schematic representation of *E. coli* ribonucleotide reductase. Protein R1 contains the catalytic site with its active thiols and two kinds of allosteric sites, one regulating the activity of the enzyme, the other its substrate specificity. Protein R2, with its two diferric Fe(III) centers and one or two tyrosyl radicals, provides the protein radical required for the reaction.

1) The activity site binds adenosine triphosphate (ATP) and deoxy-ATP (dATP) (originally called l-site because of a relatively low affinity for dATP). With ATP bound, the enzyme is active, with dATP bound it is inactive.

2) The substrate specificity site binds ATP, dATP, deoxythymidine triphosphate (dTTP), and deoxyguanosine triphosphate (dGTP) (originally called the h-site with a high affinity for dATP). With ATP bound, the enzyme reduces CDP and uridine diphosphate (UDP), with dTTP it reduces GDP, and with dGTP bound it reduces ADP.

The function of this allosteric control for mammalian reductases has been confirmed by many in vivo experiments (*18*). Clearly, class I enzymes have elaborated a highly sophisticated mechanism to ensure that cells maintain a balanced supply of the four deoxyribonucleotides for DNA replication.

Adenoysyl Cobalamin–Dependent Reductases (Class II Enzymes)

In 1964 Blakley, working in H. A. Barker's laboratory, reported that the activity of the ribonucleotide reductase from *Lactobacillus leichmannii* requires adenosyl cobalamin (*19*). Subsequent studies characterized this enzyme and described similarities as well as differences from the *E. coli* enzyme. Class II enzymes were found also in other microorganisms but not in higher organisms. This may explain why there was little interest in them after the first stage of investigation. The *Lactobacillus* enzyme received renewed interest with Stubbe's studies (*20*) that compared the mechanism of class I and class II enzymes, and its gene was recently cloned and sequenced (*21*).

Structure and mechanism of the Lactobacillus *enzyme.* The enzyme is a monomer of 739 amino acids, with a molecular mass of 81.9 kD (*21*). Its size is comparable to that of one of the protomers of *E. coli* R1. The amino acid sequence has no similarity with that of R1, with the exception of the COOH-terminus Cys-Glu-Gly-Gly-Ala-Cys-Pro-Ile-Lys, homologous to the R1 COOH-terminus Cys-Glu-Ser-Gly-Ala-Cys-Lys-Ile. The two redox-active cysteines in each sequence are proposed to transfer electrons to two other cysteines in the active site of the enzyme that directly reduce the substrate. Also in the *Lactobacillus* enzyme, a fifth, catalytically important cysteine was identified. Its function is postulated to correspond to that of Cys^{439} of R1—that is, to abstract a hydrogen atom from C-3′. In spite of the absence of any other apparent sequence homology, the two enzymes follow similar mechanisms that use electrons from reduced thioredoxin for the reduction of ribose. Identical chemistry also is implied from the stereospecificity of the reduction, which in both cases proceeds with retention at C-2′ (*22*). Furthermore, with the *Lactobacillus* enzyme the reaction also starts with abstraction of a hydrogen atom at C-3′ by a protein radical (*20*).

The major mechanistic difference between class I and class II enzymes lies in the methods proposed to generate the protein radical that initiates the chemistry. Adenosyl cobalamin takes over the function of the R2 protein, of its Fe(III) center and tyrosyl radical. Similar to other reactions involving adenosyl cobalmin, the homolytic cleavage of the bond between cobalt and C-5′ of deoxyadenosine generates a putative deoxyadenosyl radical. Originally, this was believed to be an intermediate between the thiols of thioredoxin and the reduction at

C-2′ of the ribose. With the advent of the tyrosyl radical and Stubbe's mechanism (Fig. 2), the deoxyadenosyl radical now takes over the function of the tyrosyl radical to generate a protein radical (probably a thiyl radical) that is directly involved in the abstraction of the hydrogen atom from C-3′.

Allosteric control of the Lactobacillus *enzyme.* The substrate specificity of this enzyme is also controlled by allosteric effectors (*23*). The protein contains a single binding site for deoxynucleoside triphosphates. This site is comparable to the h-site on class I enzymes, but its affinity for effectors is one to several orders of magnitude lower. The *Lactobacillus* enzyme reduces ribonucleoside triphosphates (*24*), and the products of the reaction thus act also as allosteric effectors. This leads to a complication in the allosteric control.

Nevertheless, kinetic experiments clearly established that the reduction of each of the four ribonucleotides is stimulated by one specific effector: cytidine triphosphate (CTP) reduction by dATP (and to a lesser degree ATP), UTP reduction by dCTP, ATP reduction by dGTP, and GTP reduction by dTTP. These effects are similar to those of the same effectors on class I enzymes, with the exception of dCTP, which shows no allosteric activity on class I enzymes.

Anaerobic Ribonucleotide Reductases (Class III)

Class I enzymes require oxygen for the generation of their tyrosyl radical (*11, 12*) and therefore do not function during anaerobiosis. Class II enzymes function in both aerobic and anaerobic organisms. However, microorganisms exist that grow anaerobically but do not manufacture the adenosyl cobalamin required by class II enzymes. One such organism is *E. coli.*

Extracts from anaerobically grown *E. coli* were prepared and incubated under anaerobic conditions. They catalyzed a rapid transformation of cytidine to deoxycytidine nucleotides (*25*). This activity was not inhibited by antibodies to R1 or R2, nor did hydroxyurea, a scavenger of the tyrosyl radical of R2, inhibit to the same extent. These early results suggested that the anaerobic extracts contained a new type of ribonucleotide reductase that did not operate through the tyrosyl radical. Furthermore, it appeared that CTP, and not CDP, was the preferred substrate for this enzyme activity, which therefore was classified as a ribonucleoside triphosphate reductase.

After purification, several dialyzable cofactors, including the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and dithiothreitol, were required for enzyme activity. Adenosylcobalamin had no effect. Most revealing was an absolute requirement for AdoMet (*26*), suggesting a relation between the new reductase and two other AdoMet-requiring enzymes involved in anaerobic metabolism, Pyruvate formate lyase (pfl) and lysine 2,3-amino mutase. The pfl enzyme catalyzes a key step in anaerobic glucose metabolism, and the mutase catalyzes the first step in lysine fermentation. On the surface, the two reactions have little in common with each other or with ribonucleotide reduction. However, the requirement for AdoMet and the fact that all three reactions proceed by radical mechanisms suggested common features. Seminal work by Knappe on pfl (*27*) and by Frey on the mutase (*28*) greatly facilitated work on the reductase.

Protein fractionation of the anaerobic *E. coli* extract resulted in the separation of three components. Two of these (originally named dA3 and dA1) were obtained as homogenous proteins (*29*) and were identified after their genes had been sequenced. The third fraction (RT) still requires purification, but a reasonable guess as to its identity and function can be made.

The dA3 component is the anaerobic ribonucleoside triphosphate reductase. The protein binds strongly to dATP-Sepharose, similarly to class I R1 proteins that are bound through their allosteric sites. This suggested the presence of a similar site on dA3. Furthermore, 30% of the 28 NH_2-terminal amino acids of dA3 and of R1 from *E. coli*, yeast, or mouse are conserved (*29*). Thus, the anaerobic reductase and R1 from class I enzymes share structural properties at the NH_2-terminus.

However, no further sequence homology is apparent in the remainder of the molecule. The anaerobic enzyme is a homodimer with a molecular mass of 160 kD, each protomer consisting of 712 amino acids (*30*). Its structure accommodates the functions of both the R1 and R2 proteins of class I enzymes. In addition to allosteric and substrate binding sites, characteristic for R1, the active enzyme contains also an iron center and an organic radical (*31*), characteristic for R2. However, the iron center is an iron-sulfur (Fe-S) cluster, unlike the oxygen-coupled Fe(III) center of R2. Also, the nature of the radical differs between the two proteins (see below). The enzyme displays an electron paramagnetic resonance (EPR) signal, characteristic of an Fe_3-S_4 cluster, but this may represent an inactive oxidation product of an original Fe_4-S_4 cluster, and thus the structure of the active cluster is not yet settled.

As isolated, the anaerobic reductase lacks activity. Activation requires anaerobic preincubation of the enzyme with NADPH and AdoMet and with the two other protein fractions, dA1 and RT. During this process the EPR signal of the Fe_3-S_4 cluster disappears and is replaced by a new signal with the hallmarks of an organic radical, suggesting that enzyme activation involves the generation of a protein radical.

One of the proteins catalyzing the activation reaction, dA1, was identified as ferredoxin (or flavodoxin) $NADP^+$ reductase from a comparison of its amino acid sequence with that of the spinach enzyme (*32*). This strongly suggests that the second protein, RT, is either ferredoxin or flavodoxin, and flavodoxin was indeed recently shown to be active in vitro. Activation of the anaerobic reductase thus requires its reduction by a flavoprotein system. The flavodoxin system also participates in the AdoMet-dependent activation of pfl that also generates a protein radical, recently localized to a glycine residue (Gly^{734}) close to the COOH-terminus of the polypeptide chain (*27*). The pentapeptide around this glycine has the sequence Arg-Val-Ser-Gly-Tyr, whereas the anaerobic reductase contains the pentapeptide Arg-Val-Cys-Gly-Tyr in the corresponding position (*30*). A second class III reductase homolog, from bacteriophage T4, contains the pentapeptide Arg-Thr-Cys-Gly-Tyr in the corresponding position. From this and other corroborating evidence (*30*), it appears likely that the protein radical formed during activation of the anaerobic *E. coli* reductase is a glycyl radical located on Gly^{681}.

In conclusion, the anaerobic reductase is an Fe-S protein that in its active form contains an oxygen-sensitive protein radical, probably a glycine radical, close to its COOH-terminus. The following tentative model can be proposed from the available structural information and by analogy with other systems: the iron cluster is reduced by the flavodoxin system that transfers electrons from NADPH. The reduced Fe-S cluster then transfers electrons to AdoMet to generate a 5′-deoxyadenosyl radical that, in turn, abstracts a hydrogen atom from Gly^{681} to generate the protein radical that is active in ribonucleotide reduction. The proposed formation of a 5′-deoxyadenosyl radical and its function in the generation of a protein radical is similar to the mechanism deduced for the adenosyl cobalamin–dependent class II reductases.

Allosteric regulation of the anaerobic E. coli *enzyme.* The molecular mechanism by which the anaerobic reductase is regulated is not as definitely known as the allosteric control of class I and class II enzymes because binding experiments with effectors have as yet not been done. However, kinetic experiments clearly demonstrate that the substrate specificity of the anaerobic reductase is governed by specific effectors (*33*).

The pure enzyme catalyzes the reduction

of each of the four common ribonucleotides at a slow rate that is increased up to tenfold by the appropriate effectors: ATP for CTP and UTP reduction, dTTP for GTP reduction, and dGTP for ATP reduction. Addition of the inappropriate effector is inhibitory; dATP is always inhibitory. These effects are remarkably similar to the allosteric behavior of class I enzymes.

Evolutionary Aspects

The existence at present of three different proteins with ribonucleotide reductase activity but with large structural and mechanistic differences poses the question of the extent to which any of these proteins is connected with the evolution of DNA. It was even suggested that the first reductase was an RNA molecule (*34*) and that the present protein reductases arose through three independent events that occurred after the emergence of DNA. In recent years RNA has indeed shown unexpected catalytic abilities; however, assuming a requirement for radical chemistry, the risk of self-destruction of an RNA reductase appears considerable. RNA lacks the versatility of the protein structure that can be used by nature to contain radicals in hydrophobic pockets.

In spite of the divergent protein structures, closer inspection reveals certain similarities between the three reductases. One explanation could be that the inherent properties of the chemistry of ribonucleotide reduction forced these similarities onto separately evolving enzymes by convergent evolution. However, another possibility is that the three proteins have a common ancestor from which they arose by divergent evolution through adaption to specific changing requirements of the environment. I will present the case for the second alternative, the evolution of three separate structures from a common protein "ur"-reductase.

My first assumption is that ribonucleotide reduction requires a radical mechanism, and my second is that DNA evolved before photosynthesis, before the advent of oxygen. Class I enzymes require oxygen for radical generation and could therefore not operate at this stage. A distinction between class II and class III enzymes is not possible on this basis. Class III enzymes generate their protein radical from AdoMet only under anaerobic conditions, and class II enzymes operate with adenosyl cobalamin both in the absence and presence of oxygen. From a chemical point of view the relatively simple AdoMet can be viewed as a forerunner to the complex adenosyl cobalamin. I therefore propose that the "ur"-reductase operated with an AdoMet-based glycyl radical mechanism, similar to present-day class III enzymes. Such a glycyl radical mechanism is used by pyruvate formate lyase, a key enzyme of anaerobic metabolism. One can speculate that this was a basic mechanism, first developed for anaerobic energy metabolism during evolution. The "ur"-reductase might then have exploited it for its own purpose.

The appearance of oxygen made the glycyl radical mechanism inoperative and required new ways for radical generation, involving large adjustments of the primary protein structure. I propose that class I and class II enzymes appeared in this way.

What evidence exists for their evolution from a class III–like enzyme, as compared with their appearance de novo? Even though most of the primary structures of the three reductases show no obvious similarities, some sequence identities are apparent in two areas of the proteins. The 28 NH_2-terminal amino acids of class I and class III enzymes represent one such area, where groups of identical amino acids are found in the same positions in reductases from anaerobic *E. coli*, aerobic *E. coli*, yeast, and mouse. It is hard to believe that this is the result of convergent evolution, in particular because no specific function is apparent for this part of the enzyme. A second area is found at the COOH-terminus of class I and class II enzymes, containing the two cysteine residues involved in electron transfer from reduced thioredoxin to redox-active thiols in the catalytic site. Also, other catalytically important cysteines of the two enzymes appear to be functionally and structurally related.

The strongest argument for divergent evolution from a common ancestor comes, however, from the allosteric regulation of the three enzymes. In each case their substrate specificity is controlled by binding of effector nucleotides to an allosteric site, a mechanism unique for ribonucleotide reduction. The general mechanism can be explained in terms of Koshland's induced-fit concept, not by changes in quaternary structure. Class II enzymes are monomers. Effector binding adapts the tertiary structures such that the "correct" ribonucleotide is bound and reduced. In this way the cell is assured of a steady supply of each of the four deoxyribonucleotides required for DNA replication through the action of a single enzyme.

The important point is not only that all three enzymes are subject to this unusual kind of regulation but that—with minor exceptions for class II enzymes—all reductases respond in an identical fashion to a given effector nucleotide, in spite of their different primary protein structures. If the enzymes had arisen independently of each other it seems unlikely that, for example, each of them would specifically require dTTP for GDP (GTP) reduction and dGTP for the reduction of ADP (ATP). It is more likely that a specific mechanism first became part of the primitive reductase and then was retained during evolution.

In conclusion, I propose that the three classes of ribonucleotide reductases have evolved from a common ancestor, in spite of large structural differences. The first enzyme arose before the advent of photosynthesis and, similar to present-day class III enzymes, used an oxygen-sensitive glycyl radical mechanism dependent on AdoMet. This enzyme acquired a unique allosteric control mechanism that today regulates the substrate specificity of all known reductases. The appearance of oxygen on Earth required new radical generating mechanisms involving major restructuring of the protein. Two oxygen-tolerating mechanisms evolved, one based on adenosyl cobalamin (class II enzymes), the other on an Fe(III)-tyrosyl radical center (class I enzymes). Certain structural and mechanistic features suggest a relation between the three classes of reductases, but the strongest argument comes from their almost identical allosteric behavior.

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