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# Ribonucleotide reductases: Substrate specificity by allostery

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ABSTRACT

Ribonucleotide reductases catalyze in all living organisms the production of deoxynucleotides from ribonucleotides. A single enzyme provides a balanced supply of the four dNTPs required for DNA replication. Three different but related classes of enzymes are known. Each class catalyzes the same chemistry using a common radical mechanism involving a thiyl radical of the enzyme but the three classes employ different mechanisms for the generation of the radical. For each class a common allosteric mechanism with ATP and dNTPs as effectors directs the substrate specificity of the enzymes ensuring the appropriate balance of the four dNTPs for DNA replication. Recent crystallographic studies of the catalytic subunits from each class in combination with allosteric effectors, with and without cognate substrates, delineated the structural changes caused by effector binding that direct the specificity of the enzymes towards reduction of the appropriate substrate.



## 1. Ribonucleotide reductases in the RNA world

Life, as we know it, requires two fundamentals: self-replication and catalysis. Today these are provided by three separate macromolecules: DNA takes care of replication, protein of catalysis and RNA occupies an intermediate position between DNA and protein. Early during evolution life must have started with only one of them, with a simpler chemical structure than today, but already with the dual ability for self-replication and catalysis. Only RNA fits these requirements and according to a predominant theory life started in an "RNA world" that then evolved into present days' DNA world [1]. The catalytic ability of RNA, together with the ability for self-replication, is a major argument in favour of a historically ancient role. A second major argument for life starting in an RNA world comes from a group of enzymes, the ribonucleotide reductases that today in all living cells transform ribonucleotides to deoxyribonucleotides [2]. One can conceive of ribonucleotide reductases as molecular fossils whose ancestors evolved several billion years ago and who during the transition of an RNA world into a DNA world began producing building blocks for a primitive DNA molecule that became a repository of genetic information. Ribonucleotide reduction involves radical chemistry [3,4] and requires a shielded environment that can only be provided by a protein but not an RNA molecule. We can from this conclude that also proteins probably preceded DNA during evolution.

## 2. All ribonucleotide reductases are radical enzymes

Ribonucleotide reduction is catalyzed by a complicated and highly sophisticated system. Today, life is not possible without it. During the bicentennial festivities for the birth of the Karolinska Institute we can in 2010 also celebrate the 50th anniversary of the discovery of ribonucleotide reductases at the Karolinska Institute. The first reductase appeared in an extract from *Escherichia coli* [5]. Together with an enthusiastic group of young co-workers, primarily Agne Larsson, Lars Thelander and Britt-Marie Sjöberg, we defined during the next 20 years largely by enzyme fractionation, the different components in the extract required for the replacement of the 2′-OH-group of ribose by hydrogen, resulting in the creation of a DNA precursor from an RNA precursor [6]. The whole story was delightfully complicated and at each turn full of surprises. Before that time, between 1950 and 1960, I had in various organisms without success looked for the fingerprints of the enzyme. I was convinced of its existence and too ignorant in organic chemistry to listen to the chemists who told me that such a reaction is not known in organic chemistry and therefore could not possibly take place in biochemistry either. They were right of course; it did not exist at that time.

Today we understand how this "impossible" chemical reaction does occur. From experiments with advanced biophysical methodology [7,8] and X-ray crystallography [9] we learnt that the reaction proceeds by free radical chemistry and that most ribonucleotide reductases themselves are "radical enzymes" with a stable free radical located to either a tyrosyl or glycyl residue of the polypeptide chain [3,4]. Ribonucleotide reductases exist in many different forms. However, all use a thiyl radical of a cysteine residue to activate the

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ribose moiety for reduction [4] and employ a complicated allosteric regulation that ensures the production of roughly equimolar amounts of deoxyribonucleotides for DNA replication [6]. I will later in detail discuss this regulation that is the main subject of my review. First I will describe some more general aspects.

## 3. Generation of the protein radical

Whereas the activation of ribose by a thiyl radical for the removal of the OH-group is identical for all enzymes, major differences appear in the way in which this radical is generated. On the basis of these differences the enzymes are divided into three classes [2], each with a different metallo-cofactor for radical generation (Fig. 1). Class I reductases exist only in aerobic eukaryotes and prokaryotes. In *E. coli* the enzyme is a complex of two non-identical protein dimers, named R1 and R2. R2 contains a non-haeme dinuclear iron centre that together with molecular oxygen oxidizes a specific tyrosine residue of R2 to create a stable tyrosyl radical required for the formation of a transient thiyl radical in R1. R1 is the catalytic subunit and contains, in addition to the thiyl radical, a pair of cysteines acting as electron donors for the reduction of the ribose. The cysteines are maintained in the reduced state by the thioredoxin system. Thioredoxin has further reductive tasks in metabolism, many discovered by Arne Holmgren and described in this series by him. R1 also contains the allosteric sites for effector binding. During each turnover of the enzyme a proton-coupled electron transfer occurs from the tyrosine of R2 over a distance of 35 Å to the catalytically active cysteine of R1 via a network of specific amino acids [10], an amazingly complicated process created by evolution.

Class II ribonucleotide reductases consist of only one monomeric or dimeric protein that corresponds to the R1 protein of class I enzymes. In place of R2, class II enzymes use adenosylcobalamin which generates a 5′-deoxyadenosyl radical by homolytic cleavage of its carbon–cobalt bond (Fig. 1). This radical has taken over the function of the tyrosyl radical of R2 and generates the active thiyl radical of the enzyme whose position in the structure of the enzyme corresponds to that of the thiyl radical of R1. Two redox-active cysteines, also they positioned as in class I, are substrates for thioredoxin and in their reduced form provide the electrons for the reduction of the ribotide. The generation of the 5′-deoxyadenosyl radical does not depend on oxygen, nor is it inhibited by oxygen. Consequently class II reductases operate in both anaerobic and aerobic organisms.

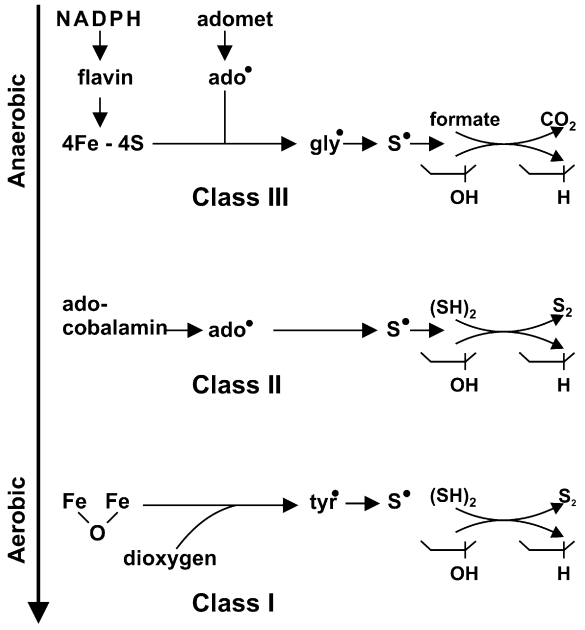


**Fig. 1.** Radical generation by the three classes of ribonucleotide reductase. In each class the ribonucleotide is activated for reduction to a deoxyribonucleotide by a thiyl radical of a catalytically active cysteine residue of the enzyme. The generation of the thiyl radical occurs by different mechanisms. In class III an "activase" uses adenosylmethionine and a reduced iron–sulphur centre to generate an adenosyl radical for the production of a glycyl radical on the enzyme that subsequently creates the thiyl radical. In class II the enzyme employs adenosyl cobalamin to generate the adenosyl radical that creates the thiyl radical. In class I one subunit of the enzyme (R2) produces a tyrosyl radical with the aid of a dinuclear iron centre and oxygen; the tyrosyl radical then generates the thiyl radical on the second subunit (R1).

Class III reductases resemble class I ribonucleotide reductases in that they require two proteins, contain a stable radical amino acid and reduce the ribotide by a thiyl radical [11]. There exist, however, major differences. Class III does not contain a separate radical storing protein. A glycyl radical forms part of the catalytic protein and is generated by a second protein, an "activase", in a reaction involving S-adenosylmethionine and a reduced iron–sulphur centre. The activase forms a 5′-deoxyadenosyl radical from S-adenosylmethionine with the aid of its iron–sulphur centre and then transfers the radical to the glycine of the catalytic protein (Fig. 1). Once formed, the glycyl radical is stable during anaerobic conditions and can carry out many reaction cycles. The radical is, however, extremely oxygen-sensitive and class III reductases therefore operate only under strict anaerobiosis. One final specific feature is that they use formate and not reduced thioredoxin as an electron donor. Class III reductases share many properties with pyruvate-formate lyase [12], a central enzyme in anaerobic energy metabolism. Both operate via a radical mechanism involving a glycyl radical and radical generation involves an activase with a reduced iron–sulphur centre and S-adenosylmethionine.

## 4. The evolution of ribonucleotide reduction

How did ribonucleotide reduction evolve during the transition of the RNA to the DNA world [13–15]? Did each of the three classes evolve independently, or do they have a common origin and then evolved further by divergent evolution? And if so, which of them came first, or rather which of them is the closest relative of a more primitive common ancestor? In sequence comparisons, one finds a limited global homology between class I and II that does not include class III. The overall structure of the active site regions, however, shows extensive homology in the architecture of all three classes. In each case the active site lies in the centre of a 10-stranded α/β-barrel with the thiyl radical at the tip of a finger loop. Interestingly also pyruvate-formate lyase has the same structural elements in its active site [12]. The common structure supports a divergent evolution from a common ancestor for all three classes as well as pyruvate-formate lyase. For the three classes the identical allosteric regulation of substrate specificity discussed below provides further evidence for divergent evolution. The different modes of radical generation summarized in Fig. 1 were later additions during evolution, caused by environmental selective pressures.

Which of today's three classes could be the closest relative to the hypothetical common primitive ancestor? [13–15]. We can exclude the oxygen-requiring class I since it is a fair assumption that DNA existed before oxygen appeared in the atmosphere. The choice then lies between class II and III, with several considerations strongly favouring the latter, among them the close relation of class III to pyruvate-formate lyase, an enzyme considered to be of evolutionary ancient origin [12]. Minerals of sulphur-linked iron were abundant on the primitive earth whereas cobalt used by class II is and was scarce. The simple S-adenosylmethionine, without cobalt, has been viewed as an evolutionary forerunner of adenosyl cobalamin [16]. Also the use of a simple molecule as formate as

external reductant, compared to thioredoxin used by class II, is in favour of a more primitive origin of class III.

## 5. Activity and specificity of ribonucleotide reductases are governed by allostery

DNA replication requires a balanced supply of all the four dNTPs. Disturbances result in increased mutation rates and may lead to disease. The dNTPs are provided by a single enzyme and their balance is achieved by the enzyme's complicated allosteric regulation. When in the early 1960s we found that ATP was required for the reduction of CDP we did not realize that we were looking at an allosteric effect nor did we understand that different allosteric effectors are required for the reduction of other ribonucleotides. At that time allostery did not yet exist. Allosteric theory was developed in Monod's laboratory [17,18] after our discovery of ribonucleotide reduction. It was introduced to explain the so called Bohr Effect, i.e. the sigmoidal binding curve of oxygen to haemoglobin studied by J. Wyman. The allosteric concept was applied by J.-P. Changeux to the feedback regulation of metabolic enzymes in which end products of a metabolic pathway inhibit the first enzyme of the pathway. The inhibitors (=allosteric effectors) are usually not structurally related to the substrate. They bind to a separate allosteric site of the enzyme and decrease its affinity for the substrate resulting in sigmoidal substrate saturation curves. Both allosteric enzymes and haemoglobin are oligomers and the allosteric inhibition was proposed to be caused by an effector-promoted change in the interaction between subunits (=allosteric transition).

When we tried to understand the peculiar requirement of the *E. coli* ribonucleotide reductase for ATP we slowly realized that we had stumbled into a new territory of allosteric regulation: the direction of the substrate specificity of an enzyme [19]. We set out to test this idea in collaboration with Neal Brown, a postdoc who had arrived from Yale. From a combination of kinetic and effector-binding data with the pure *E. coli* reductase we suggested a model for its allosteric regulation [20,21]. The conceptual foundation was that the enzyme binds effectors to two separate allosteric sites, each with a specific function: one is the usual kind of allosteric site that regulates catalytic activity (=activity site); the second site regulates the nature of the substrate to be reduced by the enzyme and thereby determines the substrate specificity (specificity site). The function of this site ensures that a single enzyme can provide the correct proportions of each dNTP for DNA replication and can rapidly adapt to changes in the requirements for dNTPs. ATP and deoxynucleoside triphosphates serve as allosteric effectors.

From the binding stoichiometry and the competition between effectors we concluded that two of them (dGTP and dTTP) only bind to the specificity site whereas the other two (dATP and ATP) bind to both the specificity and activity sites. At the activity site ATP and dATP compete with each other, with ATP stimulating and dATP inhibiting enzyme activity. At the specificity site ATP and dATP both stimulate the reduction of pyrimidine ribonucleotides, dTTP provides the specificity for GDP reduction and dGTP the specificity for ADP reduction. This general model for specificity regulation has not changed materially during the ensuing 40 years [2] and with few exceptions can be applied not only to all members of class I but also to reductases from the other two classes. Many members of class I and II lack, however, an activity site, among them many viral enzymes. But how do the specificity effectors achieve their remarkable regulatory ability?

## 6. Substrate specificity of the *E. coli* enzyme

Allosteric regulation typically is recognized from sigmoidal substrate binding curves of oligomeric proteins [17,18]. The allosteric effector changes subunit interactions affecting the binding of substrate and enzyme activity. In our case an effect of this kind may cause the effects of ATP and dATP at the activity site. It does, however, not explain changes in substrate specificity caused by effector binding at the specificity site. We proposed instead that in this case effector binding changes the local conformation of the enzyme that via its structure is transmitted to the catalytic site and there provides the signal for the binding and reduction of the appropriate substrate. This was very different from the "conventional" concept of allosteric regulation of enzyme activity via subunit interaction and our ideas did not find much favour with Monod, the high-priest of allostery. In his mind allosteric regulation required sigmoidal saturation curves, but we did not see any. The acid test was to find the hypothetical changes in enzyme structure induced by effector binding. This required X-ray crystallography.

At that time protein crystallographers did not exist at the Karolinska Institute. The only two Swedish groups worked in Uppsala at the University of Agricultural Sciences. Early on, Ingvar Lindquist, professor of inorganic chemistry, had sent two of his prize students to John Kendrew in Cambridge to learn the trade and after their return, each of them set up his own group. For quite some time Uppsala became a European center of protein crystallography. We began a long-time collaboration with Carl-Ivar Brändén's group and during the ensuing years several of his students studied various ribonucleotide reductases and determined the structural basis for the radical-driven reduction of ribonucleotides as well as their allosteric regulation.

The structure of the R1 dimer of the *E. coli* enzyme led the way [22,23]. It was not possible to solve the R1/R2 complex, but a model of it was constructed and forms the basis for the earlier mentioned calculation of the electron transfer pathway from the tyrosyl radical of R2 to the active cysteine of R1. We can, however, base our understanding of allostery on the structure of the R1 dimer alone, since the protein is the catalytic subunit of the enzyme and contains the activity and the allosteric sites (Fig. 2). The substrate site, in violet, lies in a deep cleft in the centre of each mono-

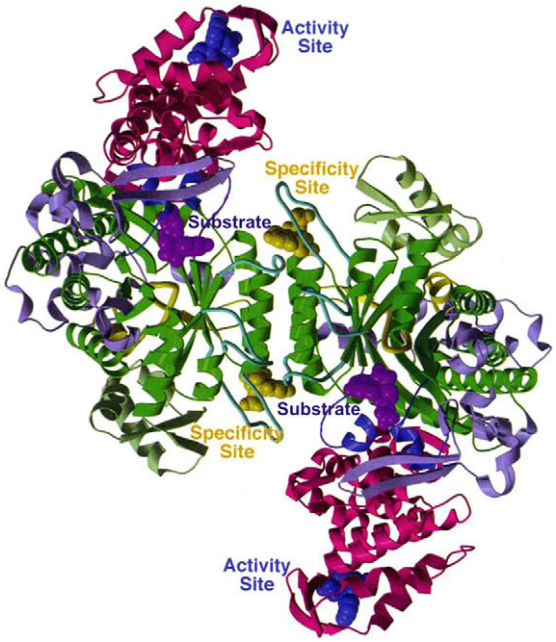


**Fig. 2.** Structure of the R1 subunit of *E. coli* (modified from [23]). Each monomer of R1 contains one binding site for substrate (violet), one allosteric activity site (blue) and one allosteric specificity site (yellow). Substrate binds in a deep cleft in the centre of each monomer, the activity effector binds at the N-terminus of each monomer, the specificity effector spans across the subunit interface of monomers with the phosphate groups bound to one subunit and the base to the other.

mer whereas the allosteric specificity site in yellow is located not too far away, spanning the subunit interface, with the phosphate groups bound to one monomer and the base bound to the other. The distance between the effector and substrate sites is too large to permit direct interaction between effector and substrate and the signal provided by the effector for the adaptation of the catalytic site to the substrate must therefore occur via the protein structure. Indeed three loops of the structure that in the absence of effector could not be located on the electron density map attained defined positions in the presence of dTTP. In particular "loop 2" appeared to be an important structural motif for specificity regulation as it forms a bridge between substrate and effector-binding sites. Finally, the allosteric activity site (blue) resides deeply in a cleft formed by the 100 N-terminal amino acids of each monomer. The *E. coli* structure thus contains separate substrate and effector-binding sites and provides evidence for conformational changes of three specific loops of the structure close to substrate and effector-binding sites implicating them in specificity signalling. A detailed analysis of the signalling mechanism could not be made as crystallization of the enzyme in combination with substrate and effector was not achieved. The structure does not explain the regulation of enzyme activity via the activity site that probably must await the crystallization of the R1/R2 complex,

## 7. Substrate specificity of the *Thermotoga maritima* reductase

A crystallographic investigation of the ribonucleotide reductase from *T. maritima*, a hyperthermophilic class II enzyme, showed the detailed structural changes responsible for the regulation of substrate specificity [2,24]. The enzyme is a dimeric protein that employs adenosyl cobalamin to generate the thiyl radical for the activation of the ribotide. In the structure, the substrate is bound as in the *E. coli* protein suggesting a similar final catalytic mechanism. Also the allosteric regulation of substrate specificity and the general binding mode of effectors are in good agreement between the two enzymes suggesting that crystallographic data obtained with the *Thermotoga* reductase are of general significance for both class I and II enzymes. We obtained good crystals of the enzyme in combination with each effector or with cognate substrate–effector complexes. In the structures of cognate effector–substrate complexes (Fig. 3) the substrate base fits into a tight pocket formed by loop 2 and a few residues from the core structure. The conformation of loop 2 is determined both by effector and substrate binding demonstrating a cooperative effect of the two nucleotides. Each effector–substrate pair comes with a distinct conformation of loop 2. The large differences for the dGTP-ADP and dTTP-GDP pairs (Fig. 3) amply illustrate this point. Loop 2 spans the specificity and catalytic sites and propagates conformational changes in the effector binding site to the substrate binding site thus providing the basis for the substrate specificity of the enzyme.

## 8. Generality of allosteric mechanism

Many of the key amino acid residues involved in nucleotide binding are conserved not only in most class II enzymes but also in class I reductases providing evidence that the rules discovered for the *Thermotoga* reductase can be generally applied to reductases from the two classes. Among the eukaryotic enzymes only the catalytic subunit of yeast has been investigated by crystallography with similar results as the *Thermotoga* reductases confirming that loop 2 also in a eukaryotic class I enzyme is at the centre of substrate specificity regulation [25]. Recently, it was found that site-directed mutagenesis of yeast loop 2 affects the composition of dNTP pools in intact cells (Kumar et al., in press) demonstrating the importance of loop 2 for the physiological regulation of substrate specificity in an eukaryote.

For class III enzymes, only the structure of the *E. coli* bacteriophage T4 reductase has been determined by X-ray crystallography [26]. The enzyme lacks the activity site usually present in class III reductases, but regulates its substrate specificity according to similar principles as class I reductases. Also in this case the X-ray structures of various effector–enzyme complexes suggested that loop 2 was closely involved in specificity regulation via an allosteric mechanism similar but not completely identical to that of classes I and II.

The appropriate composition of dNTP pools is a prerequisite for the function of living organisms. By now ample evidence exists that the allosteric control of the substrate specificity of ribonucleotide reductases plays a decisive role in pool regulation. The biochemical details of this process were worked out in vitro and found to operate according to identical principles for the three enzyme classes that evolved during evolution. The results from these experiments explain the disturbances of dNTP pools both in cultured cells after addition of single deoxynucleosides and in the body of patients suffering from immunodeficiencies [27]. Mutations in loop 2 provide a final link between molecular understand-

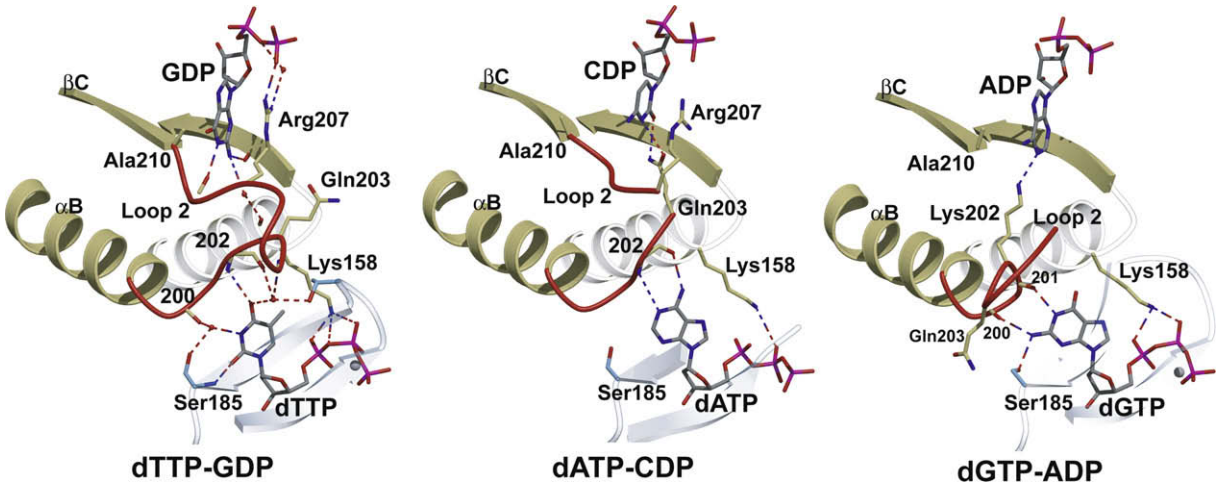


**Fig. 3.** Structures of effector–substrate complexes of *Thermotoga maritima* ribonucleotide reductase (modified from [24]). The three structures represent complexes for cognate catalytically active substrate–effector pairs. Loop 2 (in red) spans the effector and substrate binding area and obtains a different distinct conformation with each effector–substrate pair. It propagates the conformational changes caused by effector binding to the substrate site.

ing and physiological function. I believe that also Monod in his heaven now accepts that allosteric mechanisms regulate the substrate specificity of ribonucleotide reductases.

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