Ribonucleotide reductases catalyze in all living organisms the production of the deoxyribonucleotides required for DNA replication and repair. Their appearance during evolution was a prerequisite for the transition from the “RNA world,” where RNA sufficed for both catalysis and information transfer, to today’s situation where life depends on the interplay among DNA, RNA, and protein. Three classes of ribonucleotide reductases exist today, widely differing in their primary and quaternary structures but all with a highly similar allosteric regulation of their substrate specificity. Here, I discuss the diversities between the three classes, describe their allosteric regulation, and discuss the evidence for their evolution. The appearance of oxygen on earth provided the likely driving force for enzyme diversification. From today’s characteristics of the three classes, including their allosteric regulation, I propose that the anaerobic class III reductases with their iron–sulfur cluster and the requirement for $S$-adenosylmethionine for the generation of a glycyl protein free radical are the closest relatives to an ancestor ribonucleotide reductase.

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My work concerning the regulation of ribonucleotide reductases started back in the 1960s. This was the golden age of enzyme purification when many intricate regulatory mechanisms were unraveled, some of them still found in today’s textbooks of biochemistry. Inter-
the prototype for one group of aerobic reductases, named class I enzymes. Many prokaryotic reductases fall into this class, as do those from all higher eukaryotes. In procaryotes two additional classes can be found (2): class II enzymes that operate during both aerobic or anaerobic conditions and class III enzymes that function only during strict anaerobiosis. I will now briefly describe the differences and similarities between the three classes.

THREE ENZYME CLASSES

Classification depends both on differences in primary and quaternary protein structures and on functional differences (2). All ribonucleotide reductases, irrespective of class, employ free radical chemistry and to this purpose use a thiyl radical formed by a cysteine residue of the polypeptide chain (6). A major difference between the three classes concerns the generation of this thiyl radical.

Class I enzymes consist of two separate, tightly bound proteins: one large homodimeric protein with the potential to form the thiyl radical and a second smaller protein with a stable tyrosyl radical and an oxygen-linked diferric center. During catalysis the tyrosyl radical of the small protein transiently generates the thiyl radical on the large protein (7). The diferric center of the small protein is involved in the oxygen-dependent formation of the tyrosyl radical. Once formed, the tyrosyl radical is stable for many days.

Class II enzymes consist of a single protein, usually in the form of a homodimer, that is functionally equivalent to the large protein of class I. Even though the primary amino acid sequence of class II enzymes is very different from that of class I enzymes (but see below) it contains a cysteine with a potential to form a transient thiyl radical. But now the large protein—and not the small protein—contains the stable amino acid radical, which in this case is a glycyl radical instead of the tyrosyl radical of class I (8). The small protein is an "activase" that generates the stable glycyl radical on the large protein with the aid of the iron–sulfur cluster and S-adenosylmethionine (9). Once this has been achieved, the activase is no longer required for catalysis (10). Thus the large protein alone can be considered to be the ribonucleotide reductase proper of class III enzymes.

Another difference between classes concerns the reductants used for the reaction (2). Class I and class II use for this purpose the dithiols of small proteins (thioredoxin or glutaredoxin), whereas class III employs formate. Finally, the phosphorylation level of the ribonucleotide substrate provides a distinction: class I and most class II enzymes use diphosphates, whereas class III enzymes use triphosphates.

From an evolutionary point of view the relation of dioxygen to radical generation is of interest. Class I enzymes require oxygen to generate the tyrosyl radical and function therefore only under aerobic conditions. In contrast, the glycyl radical of class III enzymes is rapidly destroyed by oxygen and these enzymes therefore require strict anaerobiosis. Class II enzymes neither require oxygen nor are hurt by it. They are found in both aerobic and anaerobic organisms.

Table I summarizes some of the properties and differences between the three classes. There, class I is subdivided into class Ia and Ib. The distinction depends on differences in primary structure as well as in allosteric regulation (11).

At first sight the three classes appear very different. The amino acid sequences of the large proteins from each class show no or very few similarities. The various
classes use widely different mechanisms for radical generation. Why does a fundamental metabolic process that in all probability was a requirement for the appearance of DNA during evolution show so many different faces? However, at the nucleotide level all enzymes catalyze the same radical chemistry. And also the allosteric regulation of the three classes is very similar.

ALLOSTERIC REGULATION OF ISOLATED ENZYMES

A specific feature of ribonucleotide reduction is that a single protein reduces all four common ribonucleotides and that substrate specificity is regulated by allosteric effects. This applies to all three classes (2). In addition, also the overall activity of most class Ia and class III enzymes is modulated allosterically.

Experiments concerning the allosteric regulation of all classes of reductase (5, 11–13) involved (i) studies of the influence of effectors (ATP and deoxynucleoside triphosphates) on the rate of reduction of each of the four ribonucleotides and (ii) measurements of effector binding to the proteins.

The early E. coli work resulted in the proposal that the large protein contains two separate allosteric sites, one regulating overall activity and the other regulating substrate specificity (see Fig. 1). The former (= activity site) binds ATP and dATP, with ATP stimulating and dATP inhibiting catalytic activity. The other (specificity site) has the ability to bind dGTP and dTTP in addition to ATP and dATP. Binding of ATP or dATP to this site directs the enzyme toward reduction of CDP and UDP, binding of dGTP stimulates reduction of ADP and binding of dTTP stimulates reduction of GDP (Fig. 1). One polypeptide of the enzyme can bind two molecules of ATP or dATP, but only one molecule of dGTP or dTTP.

It was a great moment when Hans Eklund's group in Uppsala unraveled the structure of the E. coli reductase in combination with substrate and allosteric effectors (14). Now we could see that the protein indeed has the two postulated allosteric sites confirming the correctness of the general model (Fig. 2).

Effectors bound to the allosteric specificity site are at approximately 15 Å distance from the catalytic site. How do they direct substrate binding over this distance? Generally speaking, they operate by long-range interaction and affect the conformation at the catalytic site in a highly specific manner to adapt it for the "correct" substrate. A detailed understanding of this process is eagerly awaited.

With the exception of some enzymes induced by DNA viruses all ribonucleotide reductases show the same specificity pattern as the E. coli Ia enzyme (outlined in Fig. 1), suggesting that all enzymes are regulated via a similar allosteric specificity site. Binding experiments as described in (ii) above suggest that this is indeed the case for class I and II enzymes. The primary structures of these enzymes (13) contain the same strategically placed amino acid residues that form the allosteric specificity site in the structure of the E. coli Ia enzyme (14).

Class III enzymes show in kinetic experiments the same effector-dependent substrate specificity as the other two classes (12). Binding experiments suggest again the presence of two separate allosteric sites but now with a binding specificity slightly different from that of the E. coli Ia reductase. Figure 3 compares effector binding for the three classes and shows that class III binds two dATP, but only one ATP per polypeptide chain. To satisfy the results of the kinetic experiments as well as the binding experiments we proposed a model for class III enzymes in which each of the two allosteric sites regulates both the general activity and the specificity of the enzyme. When ATP binds to one site (pyrimidine site) the enzyme reduces...
pyrimidine ribonucleotides. When dGTP or dTTP bind to the other site (purine site) the enzyme reduces ATP and GTP, respectively. Binding of dATP to either site is inhibitory. Formally, the pyrimidine site of class III corresponds to the activity site of class Ia and the purine site to the specificity site (see Fig. 3). Note that despite this difference, the physiological end result is the same for the two classes.

Figure 3 also shows models of class Ib and class II enzymes. These have lost the inhibition by dATP. In most cases (all class Ib and many class II) their primary structures lack the sequence at the amino-terminus which in E. coli harbors the allosteric activity site. Binding experiments confirm that these enzymes only bind one ATP and one dATP per polypeptide. A few other members of class II have not lost the amino-terminus, bind two dATP per polypeptide, and are not inhibited by dATP (13). Also a few class Ia enzymes show this behavior. In these cases we assume that the inhibitory dATP signal is not properly transmitted to the catalytic site.

IN VIVO EVIDENCE FOR ALLOSTERIC REGULATION

The allosteric control of ribonucleotide reductases permits a rapid adjustment of pool sizes of the four
Life as we know it today depends on three classes of macromolecules: proteins, DNA, and RNA. Their interplay provides the two characteristic requirements of all living forms: self-replication and catalysis. Very early during evolution there must have been simpler scenarios, e.g., the "RNA world." RNA alone is able to transfer information and also has catalytic ability. The transition from an RNA world to present day's situation must have occurred stepwise and in one of these steps DNA replaced RNA for the storage of information. This required building blocks for DNA synthesis and gave the cue for ribonucleotide reduction in the chemical evolution of life. The fact that today all organisms from the hyperthermophilic archaebacteria to man synthesize deoxyribonucleotides by reduction of ribonucleotides strongly suggests that a primitive ribonucleotide reductase was a prerequisite for DNA replication during evolution. A corollary to this is that proteins preceded DNA during evolution (18).

The existence at present of three ribonucleotide reductases with large structural differences and with different mechanisms for radical generation poses two major questions: (i) Did today's enzymes evolve independently three times during evolution and are their similarities the result of convergence caused by the chemistry of the reaction? Or did they arise from a common ancestor by divergent evolution driven by environmental pressures? (ii) If evolution occurred by divergence, which of the three classes is most closely related to the primitive "ur"-reductase?

The amino acid sequences of the original prototypes for the three classes (E. coli class Ia, Lactobacillus leichmannii class II, and E. coli, class III) show no global similarities. This may suggest convergent evolution. However, L. leichmannii turned out to be atypical for class II and when several other class II reductases were sequenced their primary structures showed distant relationships to class I enzymes. Several other properties of the three classes also suggested a common origin, not the least the similarity of the allosteric regulation (18). All reductases respond in the same way to a given effector nucleotide, despite differences in primary structure. The mechanism for these effects is not known but considering the large distance between the allosteric and catalytic sites (Fig. 2) it must involve specific long-range interactions that affect the conformation at the catalytic sites. If the enzymes had evolved independently it seems unlikely that, for example, each would specifically require dTTP for reduction of guanine ribonucleotides and dGTP for reduction of adenine ribonucleotides. It seems more likely that a specific mechanism was part of the first reductase and was retained during evolution. In addition to the specificity site, class Ia and class III and some class II enzymes also contain a second allosteric site with the ability to bind ATP and dATP. All these proteins retain many amino acids that are part of this site, again suggesting a common origin for the three classes.

An additional argument for divergent evolution comes from the common mechanism of substrate activation by a thyl radical and the subsequent reduction of the ribose moiety by dithiols for class I and class II enzymes. At first the evidence for class III enzymes appeared less compelling as in their case formate, and not dithiols, provides the reducing power (2). However, more recently the X-ray structure of the coliphage T4 class III reductase (19) clearly demonstrated the close similarity between this structure and the class I reductase from E. coli, providing compelling evidence for a
common origin also for class III and class I enzymes. We may therefore now safely conclude that the three classes of ribonucleotide reductases arose by divergent evolution from a common ancestor and now discuss the relation of the three classes to the ancestor.

**WHICH CLASS IS THE OLDEST?**

What can be the reason for the existence today of three different classes? A possible answer lies in their relation to oxygen (18). Ribonucleotide reduction started early during evolution, before oxygen appeared in the atmosphere, and the first reductase therefore had to be an anaerobic enzyme. This excludes class I enzymes and suggests that of the ribonucleotide reductases known today either class II or class III enzymes are the closest relatives of the ur-reductase. Both classes of enzymes are prevalent in both archeae and bacteria indicating their existence before the divergence of the two domains of life. Class I has so far been found in only one single archaebacterial species (Halobacterium sp.).

In my opinion there are several reasons that suggest that a class III enzyme preceded a class II enzyme during evolution. One argument comes from the close relationship between class III and pyruvate formate lyase (pf1). pf1 is considered to be an evolutionary old enzyme. It provides a central function during anaerobic intermediary metabolism (20), the production of acetyl-CoA, a function that pyruvate dehydrogenase fulfills in aerobic cells. Similar to class III reductases, pf1 employs a glycy1 radical, formed by an "activase" and S-adenosylmethionine. Also the three-dimensional structure of the two enzymes is closely related. Clearly both have a common evolutionary origin (19, 21). It is reasonable to assume that during evolution pf1 existed before ribonucleotide reduction and that class III, and not class II, then evolved from pf1.

Other indirect arguments come from the radical generating mechanism. The combination of an iron–sulfur cluster and the relatively simple S-adenosylmethionine of class III represents a general method in anaerobic metabolism to generate carbon-centered radicals for the activation of difficult chemical reactions (22), whereas adenosylcobalamine used by class II is a complicated, highly specialized molecule. Also the use of the simple formate as reductant speaks in favor of class III, when compared to the more complicated protein-based redoxin systems.

Finally, and appropriately in this context, one can use the allosteric regulation as an argument in favor of class III. As described above, the regulation of substrate specificity follows the same rules for all reductases. However, class I and class III achieve the same end result in a slightly different way. The basic difference is that class III binds ATP only to one site (=site 1 in Fig. 4, the activity site of class Ia). In class III enzymes site 1 regulates the reduction of pyrimidine ribonucleotides, with ATP stimulating and dATP inhibiting the reactions. Site 2 of class III fulfills a similar function for purine ribonucleotides. Each site thus regulates both the specificity and the activity of the enzyme. Class Ia, instead, has divided the labor: site 2 regulates exclusively substrate specificity, site 1 exclusively enzyme activity. Class II reductases follow class Ia in their regulation of substrate specificity.

The scheme in Fig. 4 shows my hypothesis for the evolution of the allosteric regulation. Regulation started in class III enzymes with two separate allosteric sites: site 1 for the regulation of pyrimidine ribonucleotide reduction and site 2 for the regulation of purine ribonucleotide reduction. Class Ia (or class II) evolved from this situation: site 2 has now attained the ability to bind ATP and regulates all substrate specificity; site 1, instead, regulates the overall activity. Later on in evolution, site 1 became nonfunctional in some class II enzymes or was lost completely in other class II and in all class Ib enzymes.

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3 Abbreviations used: pf1, pyruvate formate lyase; CoA, coenzyme A.
controlled by allostery. This occurs by loss of the structural basis for site 1 (class Ib and some class II enzymes) or by site 1 losing the ability to transfer the appropriate signal to the catalytic site (some class II enzymes). This scenario is of course highly speculative but explains in a simple way how the present day situation may have arisen during evolution.

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REFERENCES