Allosteric Control of Three \( \text{B}_{12} \)-dependent (Class II) Ribonucleotide Reductases

IMPLICATIONS FOR THE EVOLUTION OF RIBONUCLEOTIDE REDUCTION

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Three separate classes of ribonucleotide reductases are known, each with a distinct protein structure. One common feature of all enzymes is that a single protein generates each of the four deoxyribonucleotides. Class I and III enzymes contain an allosteric substrate specificity site capable of binding effectors (ATP or various deoxyribonucleoside triphosphates) that direct enzyme specificity. Some (but not all) enzymes contain a second allosteric site that binds only ATP or dATP. Binding of dATP to this site inhibits the activity of these enzymes.

X-ray crystallography has localized the two sites within the structure of the Escherichia coli class I enzyme and identified effector-binding amino acids. Here, we have studied the regulation of three class II enzymes, one from the archaebacterium Thermoplasma acidophilum and two from eubacteria (Lactobacillus leichmannii and Thermotoga maritima). Each enzyme has an allosteric site that binds ATP or various deoxyribonucleoside triphosphates and that regulates its substrate specificity according to the same rules as for class I and III enzymes. dATP does not inhibit enzyme activity, suggesting the absence of a second allosteric site. For the L. leichmannii and T. maritima enzymes, binding experiments also indicate the presence of only one allosteric site. Their primary sequences suggest that these enzymes lack the structural requirements for a second site. In contrast, the T. acidophilum enzyme binds dATP at two separate sites, and its sequence contains putative effector-binding amino acids for a second site. The presence of a second site without apparent physiological function leads to the hypothesis that a functional site was present early during the evolution of ribonucleotide reductases, but that its function was lost from the T. acidophilum enzyme. The other two \( \text{B}_{12} \) enzymes lost not only the function, but also the structural basis for the site. Also a large subgroup (Ib) of class I enzymes, but none of the investigated class III enzymes, has lost this site. This is further indirect evidence that class II and I enzymes may have arisen by divergent evolution from class III enzymes.

Among the fascinating properties of ribonucleotide reductases is their allosteric regulation of substrate specificity (general review in Ref. 1). These enzymes balance the reduction of ribonucleotides in a way that satisfies the cell’s need for all four building blocks required for DNA synthesis. One protein catalyzes four separate reactions. The substrate specificity of the catalytic site for a given ribonucleotide is determined by binding of a specific deoxyribonucleoside triphosphate (dNTP) or ATP to an allosteric site (substrate specificity site). Thus, binding of ATP or dATP induces activity toward pyrimidine ribonucleotides; binding of dTTP induces activity toward guanine ribonucleotides; and binding of dGTP induces activity toward adenosine ribonucleotides. These effects are largely the same for all ribonucleotide reductases studied so far, except for some viral enzymes (2). Many reductases have, in addition, a second allosteric site (activity site) that regulates their overall activity, with ATP promoting and dATP inhibiting enzyme activity (1). Three classes of ribonucleotide reductases occur in nature. In addition to the earlier mentioned allosteric regulation of substrate specificity, all enzymes share a similar common free radical mechanism for the reduction of ribose and contain, for this purpose, a free radical as part of their protein structure. The three classes differ in the way in which the protein radical is produced and have evolved distinct protein structures (1, 3–5).

Class I reductases, with the aerobic Escherichia coli enzyme as the prototype, contain a tyrosyl free radical and have an \( \alpha_2 \beta_2 \)-structure (6). The large \( \alpha_2 \)-dimer has been named the R1 protein (NrdA), and the small \( \beta_2 \)-dimer has been named the R2 protein (NrdB). The tyrosyl radical forms part of the R2 polypeptide, which also contains an oxygen-linked diferric center required for radical generation. This process requires oxygen, and class I enzymes function therefore only in aerobic organisms, both bacteria and eukaryotes. The R1 protein binds substrates and allosteric effectors and is the catalytic part of the enzyme. Class I has been divided into two subgroups (Ia (NrdAB) and Ib (NrdEF)) that differ from each other functionally and in their primary structures (7). In contrast to Ia, the class Ib reductases contain no allosteric activity site. The three-dimensional structures of complexes between the allosteric effector dTTP (specificity site) or the effector analog AMPPNP (activity site) and the R1 protein of the E. coli class Ia reductase have been solved, and the amino acid residues involved in the binding of each effector were identified (8).

The signum of class II reductases (NrdJ proteins) is their dependence on adenosylcobalamin, which acts as a radical generator and thus supplies the same function as the R2 protein of class I reductases. All class II reductases contain a single

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polypeptide chain, functionally related to the R1 protein of class I enzymes. They are widely spread among aerobic and anaerobic bacteria and do not depend on oxygen. The enzyme from *Lactobacillus leichmannii* (9–11) was, for a long time, the only well characterized member of this class and became its prototype. When, in recent years, the amino acid sequences of many more class II enzymes were determined, it became apparent that the *L. leichmannii* enzyme has an unusual sequence, different from those of most other class II as well as class I and III enzymes.

Class III reductases (NrdDG proteins), with the anaerobic *E. coli* reductase as a prototype (12), have again an αβ2-structure. The catalytic α-subunit carries not only both substrate and allosteric sites, but also the free radical required for catalysis, which, in class III reductases, is located on a glycyl residue at the C terminus of the polypeptide chain. The β-subunit generates this radical with the aid of an iron-sulfur cluster in an anaerobic reaction that requires reduced flavodoxin and [2Fe-2S]-adenosylmethionine (15). The glycyl radical is oxygen-sensitive, and class III reductases can therefore operate only in strict anaerobic bacteria or facultative aerobic bacteria growing in the absence of oxygen.

Within one class, the large α- and small β-subunits each share homologous amino acids. Between the classes, however, the α-subunits of the three prototypes for the classes show only very limited homology, suggesting no or only a distant evolutionary relation between the classes. However, recent studies of class II enzymes with deep evolutionary roots demonstrated that these enzymes and class I enzymes share homologous amino acids for effector binding at critical positions in the substrate specificity site (14). As for class III, excepting the T4-induced enzyme (15), the N termini of the α-peptides containing the hypothetical allosteric second dATP site show considerable homology to corresponding segments of some of the new class II enzymes as well as to that of the *E. coli* class I enzyme.

The allosteric properties of several class I reductases (16, 17) and one class III reductase (18) have been investigated in detail by kinetic and effector binding experiments. Our knowledge of the allosteric behavior of class II enzymes is, however, incomplete. We know next to nothing concerning the recently described archaean and deeply rooted eubacterial enzymes, and early studies of the *L. leichmannii* enzyme (19, 20) need to be complemented in the light of recent knowledge. Here, we describe our experiments with three class II reductases: the previous prototype enzyme from *L. leichmannii* (9, 10), the archaean thermophilic enzyme from *Thermoplasma acidophilum* (21), and the eubacterial hyperthermophilic enzyme from *Thermotoga maritima* (14). Both *T. acidophilum* and *T. maritima* are deeply rooted organisms. *L. leichmannii*, instead, is a highly specialized microorganism, adapted to life in milk. The three enzymes had the opportunity to diverge extensively during evolution. Despite this, their substrate specificity was regulated in a similar manner, according to the same rules as for class I and III reductases. The thermophilic organisms showed a clear allosteric regulation only at elevated temperatures, suggesting that the proteins at lower temperature are not sufficiently flexible to transmit the conformational change resulting from effector binding to the catalytic site. None of the three enzymes was inhibited by dATP, and they all thus lack a functional activity site. However, the *T. acidophilum* reductase (but not the other two enzymes) meets the structural requirements for an activity site, and in effect, it did bind a second dATP. Our results will be discussed in light of a previously suggested model for the evolution of ribonucleotide reduction.

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**Experimental Procedures**

**Materials**

Bacterial strains overproducing the *L. leichmannii* (10) and *T. acidophilum* (21) enzymes were kindly provided by Dr. J. Stubbe. The *T. maritima* ribonucleotide reductase was purified from an overproducing *E. coli* strain prepared earlier in this laboratory (14). Labeled nucleoside di- and triphosphates were obtained from Amersham Pharmacia Biotech. They were diluted to the following specific activities: 15C-labeled substrates, 0–6 cpm/pmol; H-labeled substrates, 10–20 cpm/ pmol; and H-labeled effectors, 200–450 cpm/pmol. Their radiopurity was checked by chromatography on polyethyleneimine strips. If necessary, they were purified by chromatography on DEAE-Sephadex with a volatile buffer. Small portions of the dissolved labeled nucleotides were stored at −80 °C.

**Purification of Ribonucleotide Reductases**

**Common Procedures**—Batch cultures of bacteria were grown at 37 °C in Luria-Bertani broth with 100 μg/ml ampicillin to a final OD600 of 0.5, at which point isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.8 mM. The bacteria were incubated for another 3 h, harvested by centrifugation, and stored frozen. Except where indicated, all further procedures were done at close to 0 °C. Extracts of the bacteria were prepared as described earlier (14) with the exception that streptomycin (at a final concentration of 1%) was included in the extraction buffer. This greatly facilitated centrifugation of the otherwise highly viscous extracts. The clear supernatant solutions were precipitated with solid ammonium sulfate to 50% saturation. After centrifugation, the precipitate was dissolved in buffer A (30 mM Tris-HCl, pH 7.5, and 2 mM DTT) and freed from ammonium sulfate by dialysis against the same buffer. The final solution contained ~10 mg of protein/ml. This procedure was applied to 3 g of bacteria for the purification of the *L. leichmannii* enzyme and to 20–50 g of bacteria for the purification of the *T. maritima* and *T. acidophilum* enzymes.

**Further Purification of the L. leichmannii Reductase—Overproduction of the enzyme was very efficient, and at this stage, we had a total of 83 mg of protein with a specific activity of 490 units/mg of protein. Half of this protein, dissolved in 2.6 ml of buffer A, was adsorbed onto a Mono Q HR 5/5 column (Amersham Pharmacia Biotech) and eluted with a 0–1 M KCl gradient in buffer A. A peak of activity was recovered at 0.23 M KCl, and the central portion of this peak (16.5 mg of protein, specific activity = 750 units/mg of protein) was used for our experiments.**

**Further Purification of the T. acidophilum Reductase—After the ammonium sulfate precipitation of the protein from an extract of 20 g of bacteria, we recovered 1.6 g of protein with a specific activity of 14 units/mg of protein. The solution was heated at 55 °C for 30 min and centrifuged, and the clear supernatant solution was precipitated with solid ammonium sulfate to 50% saturation. The centrifuged precipitate was dissolved in 2 ml of buffer B (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 5 mM DTT) and freed from ammonium sulfate by dialysis against the same buffer. The enzyme (144 mg) now had a specific activity of 46 units/mg of protein. It was adsorbed onto a Mono Q HR column and chromatographed with a 0–1 M KCl gradient. A peak of activity (8 mg, specific activity = 186 units/mg of protein) appeared around 0.2 M KCl. After concentration in Centricon 30 tubes, the protein was adsorbed onto a 10-ml column of hydroxylapatite equilibrated with 50 mM Tris-HCl, pH 8.0. Inactive protein was removed by elution with 75 mM phosphate, pH 7.5, followed by elution of the active protein (1.7 mg, specific activity = 246 units/mg of protein) with 200 mM phosphate, pH 7.5. After concentration in Centricon 30 tubes, the protein was equilibrated with 0.1 M KCl, 50 mM Tris-HCl, pH 7.5, and 10% glycerol on a 5-ml column of Sephadex G-25, giving a final yield of 0.51 mg of pure enzyme. The protein was highly "sticky," and recovery was low. It was also prepared in pure form, but again in low yield, by affinity chromatography on dATP-Sepharose as a final purification step instead of hydroxylapatite in a procedure related to the one described below for the *T. maritima* reductase. Both preparations gave a single band on denaturing gels and, when used in binding experiments, gave identical results.

**Further Purification of the T. maritima Reductase—After ammonium sulfate precipitation, the protein in buffer B (1.4 g, 10 mg/ml, specific activity = 70 units/mg of protein) was heated for 15 min at 80 °C, and the resulting precipitate was removed by high speed centrifugation. After addition of 1.15 ml of 1 M CaCl2, the solution (114 ml, 1 mg of protein/ml, specific activity = 550 units/mg of protein) was adsorbed onto a 25-ml column of dATP-Sepharose equilibrated with 0.1 M KCl, 30 mM Tris-HCl, pH 7.5, 10 mM CaCl2, and 2 mM DTT. The column was washed with 40 ml of the same buffer, and the reductase was eluted...
with 0.5 M ammonia. The combined fractions containing 29 mg of protein were neutralized with 2 M NaH₂PO₄, and the protein was precipitated overnight after addition of solid ammonium sulfate to 70% saturation. Denaturing gel electrophoresis at this stage showed only two protein bands with mobilities of ~70 and 90 kDa. The proteins corresponding to these bands could be separated by chromatography on a column of Superdex 200 (Amersham Pharmacia Biotech) equilibrated with 0.1 M KCl, 30 mM Tris-HCl, pH 7.5, and 2 mM DTT. The ammonium sulfate precipitate was dissolved in 1.5 ml of this buffer and added to the column. Elution with the same buffer resulted in the appearance of two cleanly separated symmetrical protein peaks with mobilities corresponding to the 70-kDa band. Both TM1 and TM2 had the same N-terminal sequence as the enzyme isolated from T. maritima,2 suggesting that the smaller size of TM2 was due to C-terminal processing of the enzyme.

**Assay of Reductase Activity**

Under standard conditions, all three enzymes were incubated in a final volume of 50 μl for 20 min in the presence of 0.5 mM [³H]CTP (L. leichmannii reductase) or [³H]CDP (T. acidophilum and T. maritima reductases), 100 mM DTT, 15 μM adenosylcobalamin, 30 mM Tris-HCl, pH 8.0, 100 μM dATP, and 10 mM CaCl₂ (L. leichmannii enzyme), 10 mM MgCl₂ (T. maritima enzyme), or 40 mM MgCl₂ (T. acidophilum enzyme) at 37 °C (L. leichmannii enzyme), 80 °C (T. maritima enzyme), or 55 °C (T. acidophilum enzyme). The reaction was terminated with 1 ml of ice-cold 1 M HClO₄, and the amount of [³H]dCMP formed was determined by chromatography on Dowex-50 after 10 min of hydrolysis at 100 °C. Reduction of ADP (ATP) or GDP (GTP) was assayed with the allosteric effector. After incubation, the nucleotides were transformed to nucleosides by digestion with alkaline phosphatase, and the labeled deoxyribosides were separated from the ribosides on boronate chromatography.

**Sucrose Gradient Centrifugations**

5–20% linear sucrose gradients in a total volume of 4.6 ml were prepared in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, and 2 mM DTT. In some experiments, 15 μM adenosylcobalamin and/or 100 μM dATP was present throughout the gradient. Solutions (0.2 ml) of the enzymes containing catalase as an internal marker (s₂₀,w = 11.4 S) were layered onto the gradient and centrifuged in a Beckman SW 50 rotor at 32,000 rpm for 18 h at 20 °C. After the run, the tubes were punctured at the bottom, and a total of 33–38 fractions were collected and analyzed for protein content and catalase activity. The sedimentation coefficients of the proteins were calculated from their positions in the gradient relative to the catalase marker.

**Nucleotide Binding Experiments**

The method of Ormø and Sjøberg (25) was used at +4 °C as described previously (17, 18). No dephosphorylation of any nucleotide occurred during the course of the experiments as determined by polyethyleneimine chromatography.

**RESULTS**

**Purity and Oligomeric State of the Enzymes—Enzyme Purification**

As described under “Experimental Procedures” provided large amounts of L. leichmannii and T. maritima reductases, but only limited amounts of the T. acidophilum enzyme. All three enzymes gave rise to a single band on denaturing gel electrophoresis (Fig. 1) with positions in accordance with their molecular masses of 82, 94, and 97 kDa for the enzymes from L. leichmannii (10), T. maritima (14), and T. acidophilum (21), respectively, calculated from their amino acid compositions. In the case of the T. maritima reductase, we separated two homo-

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2 A. Jordan, R. Eliasson, U. Hellman, I. Gibert, and P. Reichard, unpublished results.
also strongly stimulated by dCTP. This is an unusual effect for T. maritima effectors, but were not absolutely required. The (GTP). With the addition of ADP (ATP); and dTTP stimulated reduction of GDP (CTP); dGTP stimulated reduction of CDP (CTP); dTTP stimulated reduction of GDP (CTP); dTTP. The results are from experiments with Ca2+ ions stimulated all enzymes in the presence of allosteric effectors, but were not absolutely required. The T. maritima enzyme worked equally well with both ions, whereas Ca2+ was slightly more active with the L. leichmannii reductase and Mg2+ with the T. acidophilum enzyme. In the latter case, the optimal concentration was as high as 40 mM, whereas the other two enzymes showed the highest activity at 10 mM. In the absence of allosteric effectors, both ions were inhibitory. These results were obtained in comprehensive experiments with CDP (CTP) as substrate, but were also verified for purine ribonucleotides in some experiments (data not shown). With guanine nucleotides, experiments with Ca2+ are limited to concentrations below 5 mM, as precipitation occurs at higher values. As reported earlier, temperature optima were 55 °C for the T. acidophilum enzyme (21) and 90 °C for the T. maritima enzyme (14).

Allosteric Properties of Reductases—A summary of the modulations of the substrate specificity of all three reductases by nucleoside triphosphates is given in Table I. Table I provides selected results from extensive experiments with each reductase in which we determined the activity with each substrate at various allosteric concentrations. The effects were strikingly similar to those observed earlier with class Ib enzymes. Active effectors were ATP and the four dNTPs. Under optimal conditions and in the presence of Mg2+ or Ca2+, the following general pattern emerges for all three enzymes: ATP and dATP stimulated reduction of CDP (CTP); dGTP stimulated reduction of ADP (ATP); and dTTP stimulated reduction of GDP (GTP). With the T. acidophilum enzyme, GDP reduction was also strongly stimulated by dCTP. This is an unusual effect for dCTP. In most cases, a 5–10-fold increase in activity was seen. dATP at concentrations up to 1 mM did not inhibit CDP (CTP) reduction, in contrast to what occurs with class Ia and III enzymes. In the absence of Mg2+ or Ca2+, the effects were smaller. Then, the reaction both proceeded faster in the absence of effector and had a lower rate in its presence (data not shown). With the T. acidophilum enzyme, temperature was also an important parameter, as found earlier for the T. maritima enzyme (21). A similar result is now shown for the T. acidophilum reductase (Fig. 2). In repeated experiments, CDP reduction was stimulated by both ATP and dATP at 60 °C, but not at 40 °C. Also the reductions of ADP and GDP by the two enzymes showed a more pronounced stimulation at elevated temperatures (data not shown). With the L. leichmannii enzyme, there was no difference between 25 and 37 °C.

The E. coli aerobic ribonucleotide reductase, a class Ia enzyme, loses its requirement for Mg2+ and allosteric effectors if supplied with high concentrations of sodium acetate (27). We wished to find out if a cobalamin-dependent enzyme behaved similarly. With CTP as substrate, the L. leichmannii enzyme had a specific activity of 84 units/mg of protein without effector, 674 units/mg of protein with dATP and Ca2+, and 414 units/mg of protein with 0.4 mM sodium acetate. Addition of either dATP or Ca2+ (or both) decreased the activity of the sodium acetate-stimulated reaction. Similar results were obtained for GTP, whereas ATP reduction was not affected. These data show that also a B12 enzyme could replace allosteric effectors with high concentrations of sodium acetate.

Binding Experiments with the L. leichmannii Enzyme—Binding of isotopically labeled dATP, dGTP, or dTTP is shown in the form of Scatchard plots in Fig. 3A. The abscissa gives the number of total binding sites is obtained by extrapolation. The dissociation constants (Kd) for each ligand can be calculated from the slopes of the curves. Deviations from linearity indicate heterogeneity of sites. For the L. leichmannii reductase, linear curves were obtained with all three dNTPs, extrapolating to values close to 1, suggesting binding of one molecule of each ligand/polypeptide to a single site. The slopes give Kd values of 2 μM (dATP), 3 μM (dGTP), and 4 μM (dTTP). The results are from experiments with 10 mM Mg2+. In the presence of 10 mM Ca2+ in

### Table I

| Enzyme/substrate | Effector | None | ATP | dATP | dCTP | dGTP | dTTP |
|------------------|---------|------|-----|------|------|------|------|
| L. leichmannii   | ATP     | 10   | ND  | 10   | 10   | 10   | 15   |
|                  | CTP     | 10   | 90  | 100  | ND   | 15   | 20   |
|                  | GTP     | 15   | ND  | 60   | 25   | ND   | 100  |
| T. maritima      | ADP     | 15   | ND  | 10   | ND   | 100  | 15   |
|                  | GDP     | 15   | 100 | 100  | 5    | 30   |      |
|                  | CDP     | 10   | ND  | 30   | 15   | 10   | 100  |
| T. acidophilum   | ADP     | 40   | ND  | 35   | 40   | 100  | 20   |
|                  | GDP     | 70   | 100 | 100  | 50   | 20   | 30   |
|                  | GDPP    | 5    | ND  | 0    | 100  | 0    | 90   |

* ND, not done.

**Fig. 2. Temperature dependence of the allosteric regulation of CDP by the T. acidophilum reductase.** The enzyme (0.4 μg/tube) was incubated with CDP as substrate under standard conditions, except for the concentration of ATP (closed symbols) or dATP (open symbols) given on the separate abscissas.
place of Mg$^{2+}$, dATP was bound linearly with a lower $K_D$ of 0.4 mM (data not shown).

To determine whether a single or several different sites bound the various ligands, we tested competition of unlabeled nucleotides with binding of labeled dATP. Increasing amounts of the unlabeled nucleotides were tested together with a fixed concentration of 10 mM dATP (Fig. 3B). dCTP, dGTP, and dTTP at high enough concentrations completely prevented dATP from binding, with dGTP being most and dCTP least efficient. In contrast, up to 2 mM ATP had little effect. The data agree with a single binding site for the four dNTPs. We believe that the apparent lack of competition by ATP depends on the high concentration of dATP required for saturation of the enzyme. ATP is an efficient effector for CTP reduction (cf. Table I).

**Binding Experiments with the T. maritima Reductase**—This enzyme bound both dATP and dGTP with a much higher affinity than the *L. leichmannii* reductase, with $K_D$ values of 0.1 and 0.3 mM, respectively (Fig. 4A). The binding stoichiometry was again one molecule of dATP or dGTP/polypeptide chain, and binding was linear. In competition experiments, dCTP, dGTP, and dTTP, as well as ATP blocked binding of dATP (Fig. 4B). Of the dNTPs, dGTP appeared most and dCTP least effective in this respect, similar to the situation with the *L. leichmannii* reductase. Now also ATP wiped out dATP binding. Due to the high affinity of the enzyme for effectors, dATP was used at a 30-fold lower concentration than in the *L. leichmannii* experiment. The experiment suggests that the *T. maritima* reductase contains a single binding site for all tested nucleotides. In an additional experiment, competition between dATP and dGTP was studied quantitatively by measuring the binding of increasing amounts of labeled dATP in the presence of two fixed concentrations of dGTP. The results of such an experiment with 1.5 and 3.0 mM dGTP, respectively, are shown in the Scatchard plots of Fig. 4C. Also in the presence of dGTP, dATP binding extrapolates to one binding site, but now with higher apparent dissociation constants. Inhibitor constants for dGTP of 0.3 and 0.5 mM, respectively, can be calculated, in good agreement with the $K_D$ of 0.3 mM for dGTP found in the experiment of Fig. 3A, providing convincing evidence for binding of

![Fig. 3. Effector binding to the *L. leichmannii* reductase. A, Scatchard plots of dATP (○), dGTP (●), and dTTP (+) binding to the enzyme (37 μg/tube). The abscissa shows the number of binding sites/protein monomer; the ordinate shows the number of sites divided by the concentration of the ligand. Extrapolation to the abscissa gives the total number of sites. B, competition of increasing concentrations of ATP (△), dGTP (○), dTTP (+), or dCTP (●) for binding of [3H]dATP to the enzyme (41 μg/tube).](image)

![Fig. 4. Effector binding to the *T. maritima* reductase. A, Scatchard plots of dATP (○) and dGTP (●) binding to the enzyme monomer (1.9–3.1 μg/tube). B, competition of increasing amounts of ATP (△), dGTP (○), dTTP (+), or dCTP (●) for binding of [3H]dATP to the enzyme (2.8 μg/tube). C, Scatchard plots of [3H]dATP binding to the enzyme monomer (4.5 μg) in the presence (1.5 μM (●) and 3.0 μM △)) or absence (+) of competing dGTP.](image)
dATP and dGTP to the same site of the enzyme.

**Binding Experiments with the T. acidophilum Reductase**—The results obtained with the *T. acidophilum* reductase differed in some important aspects from those with the two previous enzymes. This reductase is a homodimer, and binding stoichiometries will be given per enzyme dimer and not, as for the two previous enzymes, per polypeptide. One dimer of the *T. acidophilum* reductase bound two molecules of dGTP and four molecules of dATP (Fig. 5A). Thus, the binding stoichiometry of the *T. acidophilum* reductase was identical to that of the *E. coli* R1 dimer that also has the capacity to bind two molecules of dGTP and four molecules of dATP. Of these, two molecules of dGTP or two molecules of dATP bind to the substrate specificity sites, and two additional molecules of dATP bound to the activity sites. However, whereas binding of dATP to the activity sites of R1 inhibits ribonucleotide reduction, the *T. acidophilum* reductase was not inhibited similarly (cf. Table I). The enzyme bound both dGTP and dATP linearly with high affinity ($K_D$ for dGTP = 0.04 μM and for dATP = 0.03 μM). The linearity of dATP binding shows that the nucleotide was bound to each of the four sites with equal affinity. This differs from the *E. coli* R1 protein, where the specificity sites have a considerably higher affinity for dATP than the activity sites.

We next tested competition by increasing amounts of unlabelled nucleoside triphosphates for binding of dATP at a fixed concentration of dATP that approached saturation (Fig. 5B). ATP could block dATP binding almost completely, whereas dTTP and dCTP decreased dATP binding only to at most 50%, suggesting that dATP and ATP share all four sites, but that dTTP and dCTP access only two of them. The results with dGTP were less clear. In Fig. 5B as well as in two additional experiments (data not shown), dGTP decreased dATP binding to between 30 and 40%, values that did not change appreciably at high dGTP concentrations. To further study competition with dGTP, we determined binding curves for dATP at either 7.5, 50 or 200 μM dGTP (Fig. 5C) in an experiment similar to the one depicted for the *T. maritima* reductase in Fig. 4C. In the latter case, there was clear competition between dATP and dGTP for a single site on the enzyme. With the *T. acidophilum* reductase, the Scatchard plot for dATP binding is concave at 50 and 200 μM dGTP and extrapolates to two sites/dimer, as compared with four sites without competitor (Fig. 5C). Identical curves were obtained at the two dGTP concentrations. The data suggest that only two of the four sites were available for dATP binding when dGTP was bound to the enzyme. Once dGTP had occupied its two sites, further addition of dATP had no effect. The curvature of the binding curve for dATP in the presence of dGTP indicates that the two sites remaining for dATP binding are heterogeneous and bind dATP with different affinities. This then may explain why dGTP in the experiment of Fig. 5B apparently competes with $>50\%$ of dATP: when two of the four sites of the dimer are occupied by dGTP, the affinity of one of the sites remaining free for dATP binding decreases, resulting in lower occupancy by dATP at the given concentration of the ligand.

In conclusion, the binding data with the *T. acidophilum* reductase suggest that the dimer contains a total of four binding sites for dATP and ATP, two of which also have the ability to bind dCTP, dGTP, and dTTP. This differs from the other two $B_{12}$ enzymes that contain a single type of allosteric site capable of binding all dNTPs and ATP, but resembles the *E. coli* R1 protein, a class Ia reductase. On the other hand, the catalytic behavior of the *T. acidophilum* reductase resembles that of the other $B_{12}$ enzymes and differs from that of the *E. coli* R1 protein in that the *T. acidophilum* enzyme is not inhibited by dATP.

**DISCUSSION**

The “textbook” model for the allosteric regulation of ribonucleotide reductase is largely relevant for class Ia enzymes, including mammalian reductases. It has its origin in early effector binding experiments with the R1 protein of the *E. coli* reductase (16) and postulates the existence of two types of allosteric sites, two each on one R1 homodimer (or one each per polypeptide chain, as shown in Fig. 6). One type regulates the substrate specificity of the enzyme, as outlined in the Introduction, by binding ATP, dATP, dGTP, or dTTP; the other regulates overall activity by binding ATP or dATP (Fig. 6). Binding of effectors results in conformational changes that are transmitted to the active site and there affect the catalytic process. The two allosteric sites interact and operate in concert. This general model has withstood major challenges during the 30
Acids are considered homologous: Leu and Ile; Glu and Asp; Lys, Arg, and His; and Tyr and Phe.

How universal is this model? To what extent do ribonucleotide reductases belonging to other classes conform with it? For class Ia reductases, three independent lines of evidence support a common model.

**Allosteric Effects on Catalysis (Criterion i)—** Where investigated, the substrate specificity of the enzymes is affected in largely the same way by a given allosteric effector. Furthermore, the activity of the enzymes is regulated positively by ATP and negatively by dATP. There are some exceptions. The *Herpesvirus* reductases appear to lack regulation completely (2); the phage T4 (28) and *Trypanosoma brucei* (29) enzymes are not inhibited by dATP.

**Binding Stoichiometries between Effectors and Enzymes (Criterion ii)—** Results from limited experiments conform with the scheme of Fig. 6.

**Structural Considerations (Criterion iii)—** In the structure of complexes between effectors and the R1 protein from *E. coli*, the activity sites were located at the far N termini, involving the 100 N-terminal amino acids (8). The specificity sites occupied a region of between 200 and 300 amino acids from the N termini and were located between the two polypeptides making up the dimer. For both sites, nucleotide-binding amino acids were identified (8). The primary structures of all class Ia reductases, except those from herpes viruses, retain these amino acids in the appropriate positions.

We now apply these three criteria to the class II reductases studied here. (i) All three regulate their substrate specificity largely according to the pattern found for class Ia reductases. The *T. acidophilum* reductase provides an exception in that it uses dCTP, together with dTTP, as a positive effector for the reduction of GTP. However, also the T4 enzyme, a class I reductase, employs dCTP (28). The class II enzymes are not inhibited by dATP, indicating that they lack a functional activity site. In this respect, they behave as class Ib reductases.

(ii) With respect to effector binding, the enzymes fall into two groups, with the *L. leichmannii* and *T. maritima* reductases forming one and the *T. acidophilum* reductase the other. The *L. leichmannii* and *T. maritima* enzymes have only one type of site that binds all nucleotides with the same stoichiometry and thus corresponds to the specificity site of class Ia and Ib reductases. The *T. acidophilum* reductase has two types, one capable of binding all nucleotides and the other reserved for ATP and dATP. Why does binding of dATP to this site not inhibit enzyme activity, as it does in class Ia reductases? A likely explanation is that the conformational change induced by dATP binding is not transmitted appropriately to the catalytic site. A related phenomenon is the temperature dependence of allosteric effects for the thermophilic reductases (Fig. 2) (14): at the lower temperature, the protein lacks the flexibility to induce the required allosteric transition at the catalytic site.

(iii) A comparison of the amino acid sequences of the class II reductases with that of the *E. coli* R1 protein shows that the *T. acidophilum* enzyme has many of the amino acids required for the activity site in the appropriate positions (see below), whereas the *T. maritima* and *L. leichmannii* enzymes lack this part of the sequence. This agrees with the presence of a separate dATP-binding site in the *T. acidophilum* enzyme, but not in the *T. maritima* and *L. leichmannii* reductases. For the specificity site, the appropriate effector-binding residues were earlier identified in the primary structures of the *T. acidophilum* and *T. maritima* enzymes, but not in the *L. leichmannii* reductase (14).

The evidence for an allosteric model for each of the three enzymes can be summed up as follows. The *T. maritima* reductase contains a specificity site according to all three criteria, but no activity site. The *T. acidophilum* reductase contains a specificity site according to the three criteria; it contains an activity site according to criteria ii and iii, but not criterion i, i.e., dATP is bound, but does not affect catalytic activity by the evidence we have obtained. The *L. leichmannii* reductase contains a specificity site according to criteria i and ii, but not criterion iii. Substrate specificity is governed by the same rules as in other reductases, but the structural basis for the specificity site is different. Note that this enzyme occupies a special position also in other respects: it is a monomer in contrast to other reductases, and it has an unusual amino acid sequence.

![Activity site](image)

**Fig. 6.** Model for effector binding to the R1 subunit of class Ia ribonucleotide reductases. The model derives from experiments with the *E. coli* protein (16). Each polypeptide of the R1 dimer contains two separate sites with distinct functions. The substrate specificity site binds dATP (with high affinity), ATP, dGTP, and dTTP; the activity site binds ATP (with lower affinity) and ATP. Effector binding to the former site regulates the specificity of the enzyme, with dATP or ATP favoring reduction of CDP or UDP, dGTP favoring ADP reduction, and dTTP favoring GDP reduction. At the activity site, ATP binding increases enzyme activity, and dATP inhibits enzyme activity.

![Alignment](image)

**Fig. 7.** Alignment of the 100 N-terminal residues of the *T. acidophilum* reductase with the large subunits of the aerobic (class Ia) and anaerobic (class III) *E. coli* reductases. The residues of the *E. coli* involved in effector binding (8) are indicated (*). Homologous amino acids present in all three sequences are **lightly shaded**, and those present in two of the sequences are **heavily shaded**. The following groups of amino acids are considered homologous: Leu and Ile; Glu and Asp; Lys, Arg, and His; and Tyr and Phe. **TA**, *T. acidophilum*. 
contains no activity site.

All three B₃ reductases thus contain a specificity site obeying the rules of the model shown in Fig. 6, and their substrate specificity is regulated in the same way as that of class I and III enzymes. What differs from the model in Fig. 6 are the activity sites. The specificity site, was maintained throughout evolution and is today found in all reductases except in some viruses. The other, the activity site, disappeared from some class I and II enzymes. In some cases, as in class Ib and many class II reductases, the loss resulted from a deletion of the N terminus. In other cases, the loss may be a result of point mutations that disturbed the transmission of the required signal from the allosteric to the catalytic site. An example of this is the T. acidophilum reductase that binds dATP without giving the appropriate catalytic response. The fact that all hitherto known class III enzymes have maintained the structural basis for this site would be in favor of their early existence in evolution.

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