Eur. J. Biochem. 82, 393–403 (1978)

The Specificity of Ribonucleoside Triphosphate Reductase

Multiple Induced Activity Changes and Implications for Deoxyribonucleotide Formation

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(Received August 3, 1977)

Ribonucleotide reductase of *Lactobacillus leichmanii*, a monomeric enzyme requiring 5'-deoxyadenosylcobalamin (coenzyme B-12), catalyzes reduction of all common ribonucleoside 5'-triphosphates but its substrate and effector specificity is not precisely known. Structure variations in the base, as in ATP analogs with N^6-dimethyladenine, unsubstituted purine, or even benzimidazole, are tolerated but lead to greatly reduced reaction rates. These substrates respond to the same effector nucleotide (dGTP) as ATP. br^{8}ATP is completely inactive as a consequence of its abnormal molecular (*syn*) conformation. XTP is not reduced at pH 7.8 but reduction is observed at lower pH values, indicating that the species with an extra negative charge in the base ($pK = 5.7$) is inactive but uncharged xanthine results in substrate activity. dTTP stimulates reduction of XTP (and also of ITP).

The uncommon deoxyribonucleoside triphosphates dUTP, dITP, and dXTP act as allosteric effectors of ribonucleotide reduction like the common ones but they usually produce intermediate, or less specific types of stimulation, or weak inhibition. The same is true for br^{8}dATP the activity of which is explained by a certain conformational flexibility of the aglycone. A complete range of deoxyribonucleotide effects from strong activation to inhibition of enzyme activity is thus observed, indicating that the apparently specific activation pattern among natural ribonucleotide/deoxyribonucleotide couples is in fact part of the protein's general capacity to interact with nucleotides. Inspection of the substituent structures has led us to postulate certain amino acid side chains, among them a carboxylate, and hydrogen bonding interactions at the substrate and effector sites. A two-sided induced-fit mechanism is suggested to explain the unusually dynamic allosteric behaviour of *Lactobacillus* reductase.

The activity of modified ribonucleotides and deoxyribonucleotides to promote enzyme-catalyzed hydrogen exchange between 5'-[5'-^{3}H]deoxyadenosylcobalamin and water has been tested. ATP analogs and XTP are active, but in contrast to all other deoxyribonucleotides known so far the effectors dXTP and br^{8}dATP are not. In agreement with our earlier studies of sugar-modified nucleotides, these results support the existence of two different deoxyribonucleotide sites for stimulation of substrate binding and for modulation of coenzyme action.

Because enzymic reduction of modified ribonucleotides by *Lactobacillus* reductase (and also by other reductases) is very slow these findings provide a rationale as to why such compounds (*e.g.* biosynthetic intermediates or phosphorylated antibiotics) will not interfere with DNA biosynthesis under normal conditions.

Ribonucleotide reductases, like DNA and RNA polymerases, belong to a group of central enzymes in nucleic acid biosynthesis which are able to accomodate the four common purine and pyrimidine nucleotides at the same catalytic site. Their specificity is primarily determined by the sugar and 5 -phosphate moieties of the substrate molecules while the structure of the bases has an effect upon individual reaction rates. In contrast, many other enzymes of nucleotide metabolism

Abbreviations. The IUPAC-IUB abbreviations are used for nucleosides and nucleotides, see *Eur. J. Biochem. 15*, 203–208 (1970), e.g. br^{8}dATP = 8-bromo-2'-deoxyadenosine 5'-triphosphate, m$^6_2$ATP = N^6-dimethyl-adenosine 5'-triphosphate.

Enzymes. Thioredoxin reductase or NADPH:oxidized thioredoxin oxidoreductase (EC 1.6.4.5); ribonucleoside diphosphate reductase or 2'-deoxyribonucleoside diphosphate:oxidized thioredoxin 2'-oxidoreductase (EC 1.17.4.1); ribonucleoside triphosphate reductase or 2'-deoxyribonucleoside triphosphate:oxidized thioredoxin 2'-oxidoreductase (EC 1.17.4.2).

are specific for one or two particular base structures. A special property of the ribonucleotide reductases (reviewed in [1]) is their allosteric regulation by nucleotides other than the substrates (usually deoxyribonucleotides and ATP), resulting in a complex network of substrate nucleotide/protein/effector nucleotide interactions, the mechanisms of which are little understood at present.

The ribonucleoside 5′-triphosphate reductase of *Lactobacillus leichmannii* is the best known of the reductases requiring 5′-deoxyadenosylcobalamin (coenzyme B-12) (for review see [2]) which are probably more widely distributed in microorganisms than previously anticipated [3]. The *Lactobacillus* enzyme has been purified to homogeneity and studied extensively by Blakley *et al.* [4,5]. Unlike other bacterial and mammalian ribonucleotide reductases, it consists of only one large polypeptide chain of molecular weight 76000. This protein structure without subunit interactions favors analysis of nucleotide/enzyme relations. The allosteric regulation pattern has been well documented [6–9]. In the absence of effector nucleotides, the reduction rates of GTP > CTP > ATP > UTP > ITP vary by a factor of 16 between the fastest and slowest substrates, but the rate differences are reduced to less than 3-fold when specific stimulation of CTP, ATP, and UTP reduction by dATP, dGTP, and dCTP, respectively, occurs.

Several modified and unnatural ribonucleotides are also reduced by *Lactobacillus* reductase, viz. tubercidin, sangivamycin, and toyocamycin 5′-triphosphate [10] as well as benzimidazole riboside triphosphate [11]; they are subject to specific stimulation by dGTP. Likewise, the reduction of ITP, which is not a normal substrate, is stimulated by dTTP. These observations not only raise questions about the structure dependence and nature of substrate/protein interactions but also about the physiological functioning of ribonucleotide reductases which are key enzymes for the supply of DNA precursors. If other ribonucleotides present in the cell (e.g. biosynthetic intermediates, or possible regulatory compounds such as N^6-isopentenyladenosine 5′-phosphates) were reduced, they could conceivably be incorporated into DNA. DNA polymerase I from *Escherichia coli*, for example, also appears to lack strict base specificity [13]. There should have developed mechanisms to prevent such errors.

In continuation of our previous studies we have investigated eight modified or unphysiological ribonucleoside and deoxyribonucleoside triphosphates and compared them with the natural reductase substrate and effector nucleotides. They were designed to evaluate the following questions. (a) Are there any limitations for a ribonucleotide to be reduced by *Lactobacillus* reductase? (b) Can base substituents provide preliminary information about the nature of base/protein interactions at the catalytic site? (c) Is there a base specificity for deoxyribonucleotide effector binding and action, and does the stimulation of substrate reduction require unique ribonucleotide/deoxyribonucleotide combinations or less specific ones? It is shown that any modification of the main nucleotide structures greatly reduces the reactivity of compounds as substrates. At the allosteric effector site(s), however, modified deoxyribonucleotides produce significant stimulation. The data indicate a group-dependent and not an individual activation pattern of substrate reduction and allow us to recognize an unusual dynamic behaviour of the enzyme protein.

MATERIALS AND METHODS

Materials

The common ribonucleotides and deoxyribonucleotides, XTP, dUTP, dITP, and the nucleosides used for preparative work (see below) were obtained from Pharma Waldhof, Mannheim, or Boehringer GmbH, Mannheim, F.R.G. They were tested chromatographically for purity prior to use. 5′-Deoxyadenosylcobalamin and the (5′-^{3}H)-labelled coenzyme were kindly provided by Dr H. P. C. Hogenkamp, Minneapolis. Thioredoxin and thioredoxin reductase of *Escherichia coli* were prepared by the published procedure [14]. All other reagents and chemicals were commercial products of highest purity available. nol/1 M ammonium acetate (5:2, system A), or on

General Methods

Spectral measurements were made with a Cary 15 recording spectrophotometer equipped with a thermostatted cell holder. Radioactivity was determined in standard liquid scintillation fluid (Unisolve 100) using a Berthold betaszint 5000 counter. Thin-layer chromatography was performed on silica gel in ethanol/1 M ammonium acetate (5:2, system A), or on cellulose in isopropanol/water/ammonia (7:2:1, system B).

Enzyme Assays

Ribonucleotide reductase of *Lactobacillus leichmannii* [4,5] (specific activity 14 μmol mg^{-1} h^{-1} = 14 units/mg) was a generous gift of Dr R. L. Blakley, Iowa City. Spectrophotometric assays [7] were run in cuvettes of 0.50-cm light path at 25.0 °C and contained, in a total volume of 0.50 ml: 2 units enzyme (unless stated otherwise), 1 μmol substrate and effector (if present), 2 nmol thioredoxin, 0.15 unit thioredoxin reductase, 0.1 μmol NADPH, and 0.2 M phosphate buffer pH 7.8 containing 4 mM EDTA. Identical mixtures were placed into both sample and reference cells to cancel out the effect of any un-

specific NADPH oxidation. The reaction was then started in the sample cell by addition of 4 nmol 5′-deoxyadenosylcobalamin (coenzyme B-12). The absorbance change at 340 nm was recorded on the 0–0.1 scale and was linear with time and with substrate or enzyme concentration at rates of $\Delta A < 0.04$/min.

A test for possible slow reduction of br^{8}ATP included, in a total volume of 0.3 ml, 21 units ribonucleotide reductase, 0.1 µmol 5′-deoxyadenosylcobalamin, 10 µmol dihydrolipoate, 2 µmol br^{8}ATP, and 1 µmol dGTP in 0.03 M dimethylglutarate buffer pH 7.3. It was incubated in the dark at 37 °C for 23 h, terminated by boiling, and then treated with 10 µg alkaline phosphatase at pH 11. Formation of 8-bromodeoxyadenosine was not visible after chromatography in system B which resolves all nucleosides present in the mixture.

Tritium exchange reactions between 5′-[5′-^{3}H]-deoxyadenosylcobalamin (spec. act. 15.5 Ci/mol) and water [8] were carried out as follows: 0.36 unit of ribonucleotide reductase was incubated in the dark with 25 nmol of the radioactive coenzyme, 15 µmol dihydrolipoate, and 1 µmol nucleotide in 0.50 ml 0.03 M dimethylglutarate buffer pH 7.3 containing 1 mM EDTA for 15 min at 37 °C. The samples were then frozen in liquid nitrogen, connected to a recipient tube cooled in liquid nitrogen, evacuated and lyophilized. An aliquot of the collected radioactive water was used for liquid scintillation counting. Four such assays were handled simultaneously and appropriate blanks (-enzyme or -nucleotide) were included.

Nucleoside Triphosphate Synthesis

Purine riboside triphosphate and N^6-dimethyladenosine triphosphate (m$_2^6$ATP) were prepared from the nucleosides by phosphorylation with pyrophosphorylchloride [15], followed by condensation with pyrophosphate in the presence of 1,1′-carbonyldiimidazole [16]. For the first step, 1 mmol of dried nucleoside was suspended in 30 ml *m*-cresol and reacted with 1.2 ml (8.5 mmol) $P_2O_3Cl_4$ for 3 h at 0–5 °C. The reaction was worked up in the cold by addition of water and immediate neutralisation. Nucleotides were obtained in 60–80% yield by lyophilisation of the aqueous phase. They were used without further purification for triphosphate formation [16]; yields of triphosphate, after DEAE-cellulose chromatography, were >80%. The compounds were tested chromatographically for purity and were rechromatographed, if necessary, prior to enzyme experiments. Spectral properties and R_F values are summarized in Table 1.

br^{8}ATP and br^{8}dATP were prepared by direct bromination of AMP and dAMP, respectively, followed by purification of the modified mononucleotides and conversion to the triphosphates. This route was preferred over direct bromination of ATP or dATP [17] because for the enzyme experiments complete separation of product and starting nucleotides (which are active enzyme effectors) had to be assured. We were unable to quantitatively separate ATP and br^{8}ATP on columns of Dowex 1X2 (Cl^-) or DEAE-cellulose (HCO_3^-). 1 mmol of AMP or dAMP was

Table 1. *Spectral and chromatographic data of modified nucleotides and reference compounds*

Compound	R_F in solvent		λ_{max} (in water) at			ε (at pH 6)
	A	B	pH 1	pH 6	pH 12	
			nm			$M^{-1} \cdot cm^{-1}$
AMP	0.23		257	259	259	14000
ATP	0.07		257	259	259	14000
br^{8}AMP	0.26		262	265	265	17000
br^{8}ATP	0.11		262	265	265	17000
dAMP	0.25		257	259	259	14000
dATP	0.08		257	259	259	14000
br^{8}dAMP	0.29		262	265	265	17000
br^{8}dATP	0.12		262	265	265	17000
Ado		0.50				
br^{8}Ado		0.63				
dAdo		0.67				
br^{8}dAdo		0.79				
m$_2^6$AMP	0.36		267	273	273	19000
m$_2^6$ATP	0.10		267	273	273	19000
purine riboside						
5′-phosphate	0.30		262	262	262	7000
5′-triphosphate	0.09		262	262	262	7000
dXMP	0.20		234, 260	247, 276	247, 276	10000, 9100
dXTP	0.08		234, 260	247, 276	247, 276	10000, 9100
dGMP	0.17		255, 277	252, 272	265	

treated with 10 ml water, saturated with bromine, for 15 h in 30 ml 1 M sodium acetate buffer, pH 4, and the reaction mixtures were separated on columns of Dowex 1X2 (formate form) by elution with formic acid (pH 2.6–2.2). Pure mononucleotides (br^{8}AMP, br^{8}dAMP) were obtained in 50% yield; they were converted to the triphosphates as described [16]. The purity of brominated monophosphates and triphosphates was tested by chromatography in system B after enzymic dephosphorylation (alkaline phosphatase at pH 11) to the nucleosides, under which conditions traces of contaminating adenosine or deoxyadenosine could have been detected (see Table 1).

2 -Deoxyxanthosine triphosphate (dXTP) was prepared by deamination of dGMP and subsequent phosphorylation, and not by deamination of dGTP for the same reasons. 250 mg (0.56 mmol) dGMP were dissolved in a mixture of 7 ml water, 0.8 ml glacial acetic acid, and 0.28 ml 2 M HCl and treated with 300 mg (4.3 mmol) of sodium nitrite; the reaction was almost complete and was terminated after 4 h at room temperature. The solution was adjusted to pH 7, diluted with water and directly applied to a column of DEAE-cellulose (DE-32). Unreacted dGMP was eluted prior to dXMP with a gradient of triethylammonium bicarbonate (0.05–0.35 M, pH 7.5). Pure dXMP was isolated in 70% yield, dried and used for triphosphate preparation under the above conditions.

RESULTS

The ribonucleotide reductase of *L. leichmannii*, which requires coenzyme B-12, can be assayed in two independent ways: (a) spectrophotometric determination of substrate-dependent NADPH consumption in the presence of thioredoxin and thioredoxin reductase [7], an assay imitating the physiological reaction; (b) nucleotide-dependent (but not necessarily substrate-nucleotide-dependent) tritium release from 5'-[5'-^{3}H]deoxyadenosylcobalamin in the presence of reduced lipoate [8], an assay based upon the close correlation of nucleotide/enzyme and coenzyme/enzyme binding. Using these two approaches it was possible to test the modified nucleotides for different modes of interaction with the enzyme.

Substrate Specificity

All ribonucleotides studied so far [10, 11] could be reduced by *Lactobacillus* reductase, albeit at low rates. We have now tested, in comparison with ATP and GTP, the riboside 5'-triphosphate of unsubstituted purine, m$^6_2$ATP, br^{8}ATP as a nucleotide in the *syn* conformation, and xanthosine 5'-triphosphate which at neutral pH possesses an extra negative charge in the xanthine ring due to its acidic pK value (pK_a = 5.7). The results are summarized in Table 2. It is seen that the two analogs derived from ATP by removal or by methylation of the 6-amino group are less active as enzyme substrates than ATP itself but their reduction is stimulated by the same effector nucleotide, dGTP. Fig. 1 shows a linear kinetic behaviour observed for purine riboside-triphosphate, m$^6_2$ATP and ATP in the presence of dGTP. The overall rate differences are a consequence of changes in both V and apparent K_m values which vary by factors of 2–10 among these compounds.

In contrast, the 8-bromo derivative of ATP is not a substrate of *Lactobacillus* reductase. Neither in the spectrophotometric assay nor by incubation with high enzyme concentration for prolonged time could any deoxyribonucleotide formation be observed. dGTP, the common effector nucleotide for ATP analogs, and the other deoxyribonucleotides were without effect. Under the latter conditions the slow reduction of benzimidazole riboside-triphosphate was unambiguously detected [11]. We conclude that this *syn* nucleotide is virtually inactive.

The reduction of XTP exhibits an unusual pH dependence which is correlated with the equilibrium

Table 2. *Substrate activity of unphysiological purine ribonucleoside triphosphates with ribonucleotide reductase of* L. leichmannii
Conditions: 8 μM coenzyme B-12, 2 mM effector (if present), pH 7.8, 25.0 °C; see Materials and Methods for further details of standard assay. The relative rate was referred to GTP reduction as 100%

Nucleotide	Reduction	Effector	Relative rate		K_m	V
			− effector	+ effector	(+ effector)	
					mM	nkat
ATP	yes	dGTP	20	100	0.22	0.397
m$^6_2$ATP	yes	dGTP	<0.1	5	0.35	0.035
Purine riboside 5'-triphosphat	yes	dGTP	<0.1	19	0.67	0.172
br^{8}ATP	no	none	0	0	–	–
XTP (pH 7.8)	no		0	0	–	–
XTP (low pH)[a]	yes	dTTP	16	36	not measurable	

[a] Extrapolated for 100% uncharged xanthine species (XTP°) from pH dependence (see Fig. 2 and text).

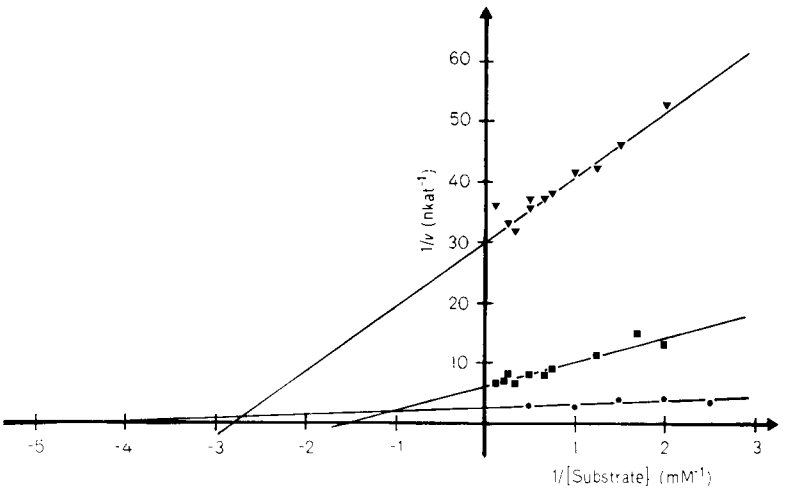


Fig. 1. *Lineweaver-Burk plot of dGTP-stimulated reduction of ATP analogs by* Lactobacillus *ribonucleotide reductase.* See Table 2 for details. (●) ATP; (■) purine riboside triphosphate; (▼) m^{2}ATP

of its XTP$^-$ (xanthine base anionic) and XTP° (xanthine uncharged) species. In Fig. 2 the rates of ATP and XTP reduction are compared at different pH values and percentage neutral (XTP°) form of XTP. While the enzyme activity decreases from pH 7.8 to 6.3 with the substrate ATP, which does not have an additional ionisation of the adenine base in this pH range, the opposite is true for XTP. At pH 7.8 (<1% XTP°) reduction is not measurable but it is clearly detected at the lower pH values (XTP° = 8% and 20% of total XTP). dTTP acts as an effector nucleotide as it does in the case of ITP. If one corrects the observed reaction rates of XTP reduction for the pH-dependent decrease in enzyme activity measured with ATP, a close parallelism between the percentage of XTP° and substrate reduction is obtained. Relative rates were calculated for 100% XTP° as enzyme substrate (16% or 36% of GTP reduction in the absence or presence of dTTP, respectively) but V or K_m values cannot be determined under these circumstances. It is thus evident that the neutral form of XTP, like GTP or ITP, is a reductase substrate while the anionic form is unable to function as such.

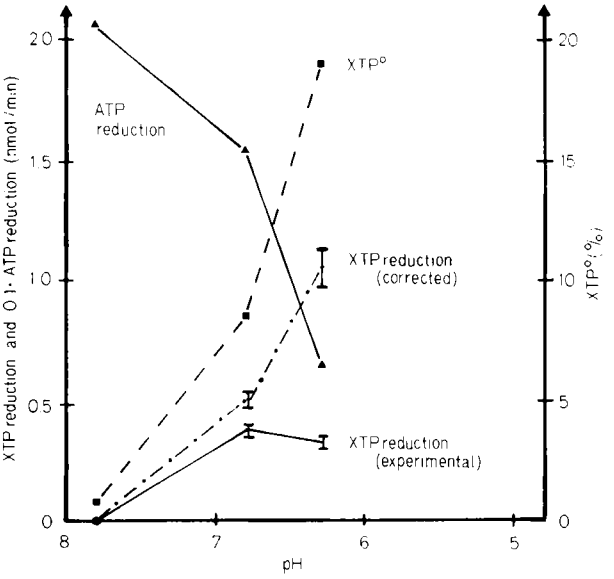


Fig. 2. *pH-dependence of ATP reduction* (▲——▲), *XTP reduction* (·····), *and percentage XTP° uncharged in the xanthine base* (■– – –■). The corrected rate of XTP reduction (–·–·–) at pH x was obtained by multiplying the experimental rate by the factor (ATP rate at pH 7.8/ATP rate at pH x)

Modified Deoxyribonucleotides as Effectors

The activation of *Lactobacillus* ribonucleotide reductase by deoxyribonucleotides appears to be the 'normal' mode of action of this enzyme under both physiological and kinetic [7] aspects. It was previously thought that the effector sites possess high base specificity but lower sugar specificity than the catalytic site [11]. On the other hand, dGTP and dTTP were found to stimulate the reduction of uncommon ribonucleotides. We have therefore prepared and tested the deoxyriboside 5′-triphosphates of xanthine and 8-bromadenine, the ribonucleotides of which were inactive as substrates. Also included were dUTP and dITP. The effects of these deoxyribonucleotides on substrate reduction rates (at saturating concentrations of all nucleotides) are summarized in Table 3. The results are expressed as percentages of the rate of GTP reduction to facilitate comparison with previous data; the stimulatory effects noted for the common ribonucleotide/deoxyribonucleotide couples were identical with those reported before [7]. It is not surprising that dUTP is able to substitute for dTTP in the stimulation of XTP and ITP reduction. More importantly, both are slightly active in stimulating UTP reduction, an effect different from the apparent product inhibition observed for other substrate/product pairs

Table 3. *Effect of deoxyribonucleoside triphosphates upon substrate reduction by* Lactobacillus *ribonucleotide reductase*
Conditions were as in Table 2. Reaction rates are expressed as percentages of GTP reduction. Bold-face figures indicate at least twofold stimulation in presence of a deoxyribonucleotide

Substrate	No addition	+ dATP	+ br^{8}dATP	+ dCTP	+ dTTP	+ dUTP	+ dGTP	+ dITP	+ dXTP[b]	
									pH 7.8	pH 6.3
ATP	20	15	20	15	17	17	**100**	**38**	20	27
CTP	36	**100**	**70**	32	32	32	41	27	43	54
UTP	14	14	14	**37**	17	17	23	6	9	9
XTP[a]	16				**36**	**33**				
ITP	6	6	**11**	7	**38**	**38**	5	5	6	9

[a] Corrected for 100% uncharged xanthine species (XTP°, *cf.* Table 2); other deoxyribonucleotides were also tested and showed no significant stimulation of XTP reduction.
[b] dXTP was present in 5 mM concentration.

Table 4. *Enzyme-catalyzed tritium release from 5'-[5'-^{3}H]deoxyadenosylcobalamin into water in the presence of nucleoside triphosphates*
Conditions: 50 µM radioactive coenzyme, 2 mM nucleotides, incubation for 15 min at 37 °C (see Materials and Methods for further details). Results are expressed as the total radioactivity of water in a 0.50-ml standard assay

Nucleotide	pH	Radioactivity
		dis./min
dGTP	7.3	602000
dGTP, no reductant	7.3	1800
dGTP, no enzyme	7.3	1600
ATP	7.8	429000
ATP	7.3	396000
ATP	6.8	317000
br^{8}ATP	7.3	4400
m_2^6ATP	7.3	24300
Purine riboside 5'-triphosphate	7.3	83900
dATP	7.3	167000
br^{8}dATP	7.3	5000
dXTP	7.3	4200
XTP	7.8	39800
XTP	7.3	35200
XTP	6.8	22500
XTP + dTTP	6.8	162000
dTTP	6.8	14700

(ATP/dATP, CTP/dCTP, and GTP/dGTP). A new activator was found with br^{8}dATP, which may replace the parent nucleotide, dATP, in a significant stimulation of CTP reduction. Because the br^{8}dATP used was virtually free from dATP its activity cannot be due to contamination. Moreover, br^{8}dATP slightly stimulates ITP reduction, an effect not produced by dATP itself. When br^{8}ATP was added to assays containing CTP as substrate, no stimulatory or inhibitory effects were seen. dITP is an effector of ATP reduction although it is less potent than the related nucleotide, dGTP. In the case of dXTP increasing stimulatory action upon ATP, CTP, and ITP reduction is noted when the pH is lowered from 7.8 to 6.3, again indicating that it is the dXTP° species with an uncharged xanthine base which acts as an effector of ribonucleotide reductase. At pH 6.3 and a dXTP concentration of 5 mM the concentration of dXTP° in the assay is approximately 1 mM. Neglecting contributions of dXTP$^-$, dXTP° must then be considered a fairly active effector nucleotide, particularly for CTP reduction where it replaces dATP while in its effects upon ATP and ITP reduction it would have to mimic dGTP and dTTP.

It is obvious from these data that the allosteric phenomena typical of *Lactobacillus* reductase are by no means limited to the main nucleotide structures but that intermediate stimulation factors (from zero to six-fold) and various substrate/protein/effector interactions occur. An attempt to unify these observations on a structural basis will be discussed below.

Tritium Exchange Experiments

The enzyme-catalysed hydrogen exchange between 5'-[5'-^{3}H]deoxyadenosylcobalamin and water in the presence of nucleotides [8] enables one to study the effect of nucleotides on enzyme-coenzyme rather than on enzyme-substrate interactions. The activity of synthetic purine ribonucleoside and deoxyribonucleoside triphosphates in this reaction is shown in Table 4.

Again the control values (standard assay + dGTP, dATP, or ATP as effectors) closely resemble previous data [8, 11]. The modified substrates, m_2^6ATP and purine riboside-triphosphate, are also active in the exchange reaction. Their activity roughly parallels the differences observed versus ATP in the kinetic assay (Table 2), just as br^{8}ATP is (almost) inactive in both reactions. In contrast, bromoadenine and xanthine nucleotides may also exhibit opposite effects in the exchange reaction and in overall ribonucleotide reduction: br^{8}dATP and dXTP promote very low tritium exchange although they are activators of substrate reduction (Table 3). Conversely, XTP at high pH, where it cannot function as a substrate, produces rather strong tritium exchange. The pH-dependence of XTP-catalysed exchange parallels that of ATP and

is not reciprocal to it as in substrate reduction (Fig. 2). These data show that anionic xanthine in this function is not intolerable for the enzyme as at the substrate site. Finally it is remarkable that in the presence of XTP plus dTTP tritium exchange is greatly stimulated beyond an addition of both nucleotide effects. Such a conflicting behaviour of nucleotides in substrate reduction and tritium exchange is not normally observed with the common ribonucleotides and deoxyribonucleotides but it has been noted before for sugar-modified analogs with intermediate activity towards *Lactobacillus* reductase [11]. Tritium exchange obviously has its own structure specificity.

DISCUSSION

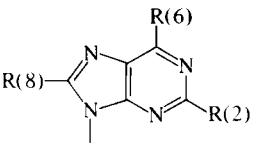
The 5′-deoxyadenosylcobalamin-dependent ribonucleoside triphosphate reductase of *L. leichmannii* has a less complex protein composition than bacterial and mammalian diphosphate reductases but it still exhibits a high degree of complexity. The monomeric protein of about 690 amino acids [4] has a catalytic site which is highly specific for the β-D-ribofuranose 5′-triphosphate moiety of substrates, specific for most corrin and nucleoside substituents of the coenzyme, and may accomodate thioredoxins or low-molecular-weight dithiols as reductants [2]. Moreover the enzyme activity is strongly modulated by allosteric effectors for which there appear to be two sites of interaction [11]. Binding of a proper compound at any of these sites affects the affinities of all other sites, reflected in numerous K_m and rate changes [2, 7]. It is impossible at present to describe this entire dynamic behaviour of the enzyme by a consistent kinetic scheme for lack of comprehensive experimental data as well as of suitable kinetic models. However, what can be attempted is to delineate the basic structures and modes of interaction which must participate in enzyme-nucleotide relations irrespective of detailed sequences of events in binding and catalysis. Such information is valuable for further, more specific, analysis of the complex enzyme system.

Substrate Site Specificity

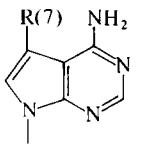
An absolute requirement for the unsubstituted 2′ and 3′-hydroxyl groups of ribose at the substrate site of *Lactobacillus* reductase has been established [11]. Relative reduction rates (GTP = 100%) for the 12 ribonucleoside 5′-triphosphates with purine or related bases studied so far are summarized in Table 5. It is evident that the substituent pattern of the aglycone determines the individual reaction rates (and also the kind of effector nucleotide). The changes of apparent K_m value observed for ATP, m_2^6ATP, and purine riboside triphosphate under identical conditions vary only by a factor of 2–3 (Table 2), indicating that these structure changes affect mostly V and that the common ribose triphosphate structure is respon-

Table 5. *Relative reduction rates of purine ribonucleotides and related analogs catalysed by* Lactobacillus *ribonucleotide reductase: structure and effector correlations*

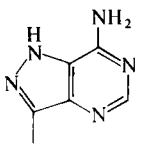
Aglycone structures:



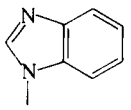
I–VII



VIII–X



XI



XII

Compound (triphosphate of)	R(2)	R(6)	R(7)	R(8)	Relative rate		Effector nucleotide	Reference
					−effector	+effector		
I. Guanosine	NH_2	O=		H	100	100	–	[7]
II. Inosine	H	O=		H	6	38	dTTP	[7]
III. Xanthosine (neutral)	O=	O=		H	16	36	dTTP	
IV. Adenosine	H	NH_2		H	20	100	dGTP	[7]
V. N^6-Dimethyladenosine	H	$N(CH_3)_2$		H	<0.1	5	dGTP	
VI. 8-Bromoadenosine	H	NH_2		Br	0	0	–	
VII. Purine riboside	H	H		H	<0.1	19	dGTP	
VIII. Tubercidin (= 7-deazaadenosine)			H		35	75	dGTP	[10]
IX. Toyocamycin			CN		1	50	dGTP	[10]
X. Sangivamycin			$CONH_2$		1	8	dGTP	[10]
XI Formycin					0	0	–	[10]
XII. Benzimidazole riboside					<0.1	2	dGTP	[11]

sible for the magnitude of K_m. Compounds with polar keto and amino substituents in the 2 and 6 positions are good substrates (I–IV, VIII) but methylation or removal of these substituents, or changes in the five-membered ring, yield poor substrates in the unstimulated reaction (V–VII, IX–XII). Under no conditions could we observe a reduction of br^{8}ATP (VI). The only other purine analog known to lack substrate activity is formycin triphosphate (XI). The change in primary structure between these two compounds and ATP is very small and can hardly be the cause of their inactivity but they have in common an abnormal three-dimensional structure: br^{8}AMP and formycin 5′-phosphate do not show the *anti* base conformation typical of other nucleotides (in which the six-membered ring or purine points away from the ribose) but they have a *syn* or intermediate base conformation due to the presence of a space-filling 8-substituent, or to lack of intramolecular dipolar structure stabilisation, respectively [18–20,32]. This finding demonstrates that *Lactobacillus* reductase has an absolute specificity for the *anti* conformation of substrate nucleotides, similar to polynucleotide phosphorylase or RNA polymerase which will not accept the substituted guanine nucleotides, br^{8}GDP and br^{8}GTP, as substrates [21,22]. Because all natural nucleotides possess the *anti* conformation, such a specificity of enzyme sites is *per se* plausible; we have recently given a general explanation of the *anti* preference in nucleotides and enzyme specificities based upon the intramolecular forces present in nucleoside molecules [23]. However, the inactivity of XTP$^-$ must have other reasons because this compound cannot adopt a *syn* conformation in which anionic phosphate and anionic pyrimidine ring would be close together.

For all the ribonucleotide substrates of Table 5 the order of reactivities remains unchanged in the absence or presence of effector nucleotides. We therefore assume that an allosteric effector in this system optimizes substrate base-protein interactions without making entirely new contributions, thus improving substrate binding and catalysis at C(2′)-OH. The following details of substrate site construction may then be deduced from an inspection of Tables 2 and 5. Hydrophobic or π-electron interactions between the bases and aromatic amino acids cannot play a significant role because unsubstituted substrates (VII, XII) and m$_2^6$ATP are least active. We postulate two types of hydrogen bonding between substrates and protein groups, one for interaction with amino-substituted nucleotides which react best in the stimulated reaction (GTP, ATP, tubercidin-triphosphate and CTP, *cf.* Table 3) and the other for interaction with somewhat less reactive, carbonyl-substituted nucleotides (XTP, ITP, and also UTP). The first protein group must be a hydrogen-bond acceptor and the other a hydrogen-donating residue. A clue to the nature of the hydrogen-accepting amino acid residue lies in the inactivity of anionic XTP$^-$, indicating that it is an anionic aspartate or glutamate residue which prevents binding of XTP$^-$ but interacts well with GTP, ATP, or CTP. Because the position of the amino group in these three substrates is different, equal interactions and reaction rates are only reached by effector-induced subtle conformation changes. The nature of the second amino acid side chain (e.g. a protonated histidine) remains to be established. In the reduction of GTP which does not require an effector both amino acids would cooperate, UTP and uncharged XTP could probably interact with one of them, and with *syn* nucleotides there should be no interaction at all.

This hypothetical situation resembles nucleotide binding by a glutamate and a histidine residue at the active site of ribonuclease T$_1$ [24], an enzyme which preferentially hydrolyses guanylic acids and reacts slowly with inosine derivatives but lacks allosteric activation. Unfortunately, due to the presence of multiple nucleotide sites our postulate is much more difficult to verify experimentally than in a small enzyme. Another complication is the requirement for thiol groups as reductant. Thus, preliminary attempts to study the interaction of *Lactobacillus* ribonucleotide reductase with 6-(2,4-dinitrophenyl)-mercaptopurine riboside 5′-triphosphate, an active-site-directed affinity reagent developed by Fasold [31], were unsuccessful because in the spectrophotometric enzyme assay this nucleotide inactivated thioredoxin reductase.

Allosteric Effectors

We had previously presented evidence for interaction of *Lactobacillus* reductase with nucleotide effector molecules at two different sites, one promoting coenzyme binding and tritium exchange, and the other stimulating substrate reduction [11]; of course under conditions *in vitro* both these sites can also be occupied by the same nucleotide. Only one nucleotide site specific for deoxyribonucleotides was observed during equilibrium dialysis of protein and various nucleoside triphosphates [9] but these experiments did not include coenzyme. Our present studies appear to substantiate the two-site model because with br^8-dATP and dXTP we have found two activators which, in contrast to all other deoxyribonucleotides tested so far, have only negligible ^{3}H exchange activity; in this respect they resemble *O*-methylated ATP analogs [11]. On the other hand XTP$^-$ efficiently catalyses tritium exchange. Although these obvious structure discrepancies are no direct proof of two effector sites in the complete system it would be extremely difficult, if not impossible, to explain the

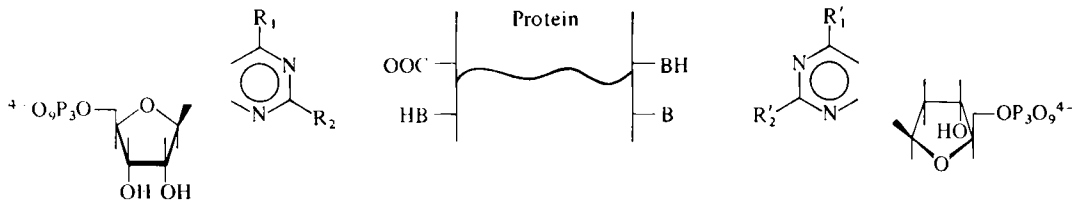


Fig. 3. *Minimum structures engaged in the allosteric modulation of* Lactobacillus *ribonucleotide reductase*. Substrate and effector nucleotide binding occurs at their sugar phosphate moieties and is optimized by two-sided induced-fit involving purine or pyrimidine base substituents R (NH_2- or O=) and protein groups. B and B-H are postulated basic, or anionic, and protonated amino acid side chains engaged in hydrogen bonds or other polar interactions with the nucleotides

effects of all analogs now available by binding to the same allosteric site. However, it is also clear that these nucleotide sites are not independent of each other but closely interconnected. This follows, for example, from the stimulation of ^3H exchange by XTP plus dTTP (a phenomenon not observed before), or from the complex types of inhibition produced by the arabino analogs of ATP and CTP in the absence and presence of activators (to be published elsewhere).

Before analysing structural details of substrate-effector relations one can recognize basic specificity elements apparently common to all nucleotide sites of the enzyme. One is the marked preference of purine (in particular, guanine) nucleotides which expresses itself in substrate reduction rates, in their more general effector activities, and in stronger tritium exchange. Furthermore, substitution or removal of purine substituents usually reduces reaction rates and hydrogen exchange activity (in ATP vs m_2^6ATP and purine ribonucleoside triphosphate, GTP and dGTP vs ITP and dITP). In view of these similarities the good effector activity of br^{8}dATP, as compared with the total inactivity of br^{8}ATP, is unexpected. Complete lack of conformation specificity at the effector site is unlikely because then br^{8}ATP should also act as an activator. No final decision can be made but the following explanation of this discrepancy is reasonable. 5′-Nucleotides of ribose and deoxyribose possess very similar three-dimensional structures but the deoxyribonucleotides tend to have slightly greater conformational freedom [25]; in connection with the much higher affinity of the enzyme for deoxyribonucleotides [9] this might result in fixation of br^{8}dATP (but not br^{8}ATP) in various states of *syn* ⇄ *anti* base rotation, in keeping with its unusual, ambiguous action upon CTP and ITP reduction. Conformation changes between free and enzyme-bound nucleotides have occasionally been invoked for polymerizing enzymes [21,22,26] but direct experimental proof, or comparative data of activation barriers for aglycone rotation, are still missing.

Probably the most important result of our present studies is the demonstration of intermediate and ambiguous cases of activation of substrate reduction (Table 3). With the exception of GTP reduction which is very little influenced by other nucleotides one can group the effects of deoxyribonucleotides upon enzymatic reduction of the common ribonucleotides into a scheme ranging from strong activation (more than 2-fold) to moderate inhibition (less than 2-fold):

Activation →			No (or little) effect	←Inhibition
ATP	dGTP	dITP, dXTP	br^{8}dATP	dATP, dCTP
CTP	dATP	br^{8}dATP, dXTP	dGTP, dTTP	dCTP, dITP
UTP	dCTP	dGTP, dTTP	dATP, br^{8}dATP	dXTP, dITP

This pattern indicates that the weak inhibition of ATP reduction by dATP, and of CTP reduction by dCTP observed before [7] must not be interpreted as common product inhibition (in agreement with the unlikely binding of deoxyribonucleotides at the catalytic site) but is only an example of the gradual transition from stimulation to inhibition shown by all deoxyribonucleotides including the uncommon ones. A general explanation of the above effects is that binding of any deoxyribonucleotide at an allosteric site produces slightly different conformation changes of the protein which express themselves in more facile, or less favorable, nucleotide-protein interactions at another nucleotide site, depending upon the structure of that other nucleotide.

One can finally recognize structural requirements for the complete pattern of molecular interactions between a substrate nucleotide at the catalytic site and its effector nucleotide(s) at the allosteric site responsible for rate stimulation. (The other allosteric site mediating coenzyme interactions cannot be included here because it has different specificity, requiring another set of analogs for final evaluation.) Fig. 3 tries to assemble the information discussed earlier for substrate-protein interaction and the action of deoxyribonucleotides described above. Obviously the 'fit' on either side of the ternary complex must involve the polar amino and keto substituents of

nucleotides. At the effector site amino-substituted nucleotides (dGTP, dATP, dCTP) usually produce good stimulation, those with two keto groups are less effective (dTTP, dXTP), and dITP with only one keto group is least active in stimulation, but inhibitory in several cases. Taken together, all these observations suggest a basically similar construction of both nucleotide sites with respect to the bases and also participation of hydrogen bonding interactions at the right side of Fig. 3. Individual reaction rates of any nucleotide combination are then determined by the total energy of possible interactions and of the appropriate conformation of the polypeptide region connecting the two sites. We name this the two-sided induced-fit mechanism of allosteric enzyme modulation.

Even without knowledge of analog binding the allosteric behaviour of *Lactobacillus* ribonucleotide reductase must be considered exceptional in that it affects a monomeric protein and the reaction of (at least) four different substrates. However the catalytic and allosteric sites are spatially arranged on the protein one has to envisage dynamic folding of the polypeptide chain capable of transmitting all the necessary interactions of substrates, coenzyme, and effectors. Our finding discussed above are strong evidence for a highly adaptable, multi-state type of allosteric modulation rather than a simple two-state model. Although this analysis of protein dynamics by substrate and effector analogs is limited to a few basic aspects, it does provide an understanding of enzyme function before protein modification or X-ray crystallography experiments can be done; these will be extremely complex in this case.

Physiological Significance

The results discussed above are primarily of interest for understanding the catalytic and allosteric mechanisms of *Lactobacillus* ribonucleoside triphosphate reductase and not so much its action in the cell. Nevertheless it is worthwhile commenting on their physiological significance because the diphosphate reductase of *Escherichia coli*, an enzyme of entirely different protein structure and cofactor requirement, possesses a basically similar mechanism and specificity [10–12,27] (and W. Ludwig and H. Follmann, unpublished results), indicating that ribonucleotide reduction as a key process of DNA replication may be subject to some general constraints.

Various modified ribonucleotides are known to occur intracellularly, such as the following examples: IMP, XMP, orotidylic acid (a nucleotide having *syn* conformation) and other metabolic intermediates of the four main nucleotides; phosphorylated forms of nucleoside antibiotics such as tubercidin, formycin, and nebularin (purine riboside) [28] (and references therein); cytokinins such as N^6-isopentenyladenosine which is converted to its diphosphate or triphosphate in plant cells [29]. Incorporation of several such nucleotides into RNA has been reported. Many of them should, in principle, also be capable of being enzymatically reduced to deoxyribonucleotides. In that case, incorporation of purine or pyrimidine bases lacking proper conformation or substituents for correct base pairing into DNA appears possible although it may be corrected by the proof-reading function of DNA polymerases. However, abnormal allosteric effects of modified deoxyribonucleotides upon ribonucleotides reductases could still strongly interfere with the balanced supply of DNA precursors. To our knowledge, natural occurrence of such modified deoxyribonucleotides, as well as incorporation of odd ribonucleotides into DNA are unknown. (An exception is the uptake of tubercidin into fibroblast RNA and DNA [30] but this is explicable by the almost identical structure of tubercidin and adenosine, making the triphosphate a good substrate of *Lactobacillus* and *E. coli* reductase [10,27].) If our results with the bacterial ribonucleotide reductases may be generalized, they provide a simple explanation of why modified ribonucleotides do not interfere with DNA biosynthesis: although one enzyme can hardly be expected to possess absolute specifity for all main nucleotides, which are of quite different chemical structure, the reductases have obviously been adapted in such a way that any abnormal exocyclic substitution or molecular conformation of a nucleotide leads to very poor, or missing, substrate activity. The greatly reduced reaction rates in ribonucleotide reduction, together with low intracellular concentrations, of 'wrong' nucleotides will efficiently protect a cell from detrimental effects of such compounds upon DNA replication.

We are greatly indebted to Drs R. L. Blakley and H. P. C. Hogenkamp (Department of Biochemistry, University of Iowa, Iowa City) for enzyme and coenzyme samples. This work is supported by a grant from the *Deutsche Forschungsgemeinschaft*.

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