Substrate and Effector Binding to Ribonucleoside Triphosphate Reductase of *Lactobacillus leichmannii*†

Alice K. Chen, Ashok Bhan, Sarah Hopper, Richard Abrams, and James S. Franzen*

ABSTRACT: Ribonucleoside triphosphate reductase, isolated from *Lactobacillus leichmannii*, is shown to be comprised of one polypeptide chain having a molecular weight of 76,000 daltons, in agreement with an earlier report (Panagou, D., Orr, M. D., Dunstone, J. R., and Blakley, R. L. (1972), *Biochemistry 11*, 2378). Direct binding studies by equilibrium dialysis and differential fluorescence methods indicate that only one site exists for binding of the enzyme effectors, the deoxyribonucleoside triphosphates. Dissociation constants for effector binding range from 10^{-4} to 10^{-5} M. Competition binding experiments substantiate the view that all the effectors bind at one and the same site. Nucleotide substrate binding was not observed by direct methods under any conditions employed. Competition for the effector site between effector and substrate was observed, however. Generally, substrate binding at the effector site was two orders of magnitude weaker than effector binding at this site. These observations suggest that ribonucleotide addition at the substrate site may be one of the last occurring events in the overall catalytic process, and that substrate may act as its own effector by binding at the effector site.

It has long been recognized that the catalytic activity of ribonucleoside triphosphate reductase from *Lactobacillus leichmannii* is subject to positive modulation by the presence of the reaction products (Goulian and Beck, 1966; Vitols *et al.*, 1967a). Beck has reported that maximal stimulation of the reduction of CTP is caused by dATP, of ATP by dGTP, of GTP by dTTP, and of UTP by dCTP (Beck, 1967). Unpublished observations from our own laboratory are in essential agreement with these effector–substrate relationships. It has been claimed that this allosterically controlled enzyme is comprised of one and only one polypeptide chain (Panagou *et al.*, 1972). Evidence to be presented here solidly confirms this conclusion. In light of the nonsubunit nature of this reductase, the nature of the mechanism which produces sigmoidal kinetics in the absence of effectors, observed as mentioned both by Beck and ourselves, is of particular interest. Generally, the appearance of such positive cooperativity in the reaction rate *vs.* substrate profile is associated with intersubunit effects in enzymes possessing quaternary structure. Lack of quaternary structure in this case rules out all such mechanisms. However, if multiple sites for substrate anchoring exist on the single properly folded polypeptide chain, one can still invoke site–site interactions yielding rate profiles of sigmoidal form. On the other hand single site random pathway models can also be developed to explain non-Michaelis–Menten-style kinetics (Fisher, 1972). The elucidation of the mechanism of substrate and effector control of the *L. leichmannii* reductase therefore is dependent on a knowledge of the number of binding sites available for substrate and effector binding, the stability of the complexes formed and the magnitude of possible homotropic and/or heterotropic interactions. Although a number of papers have appeared with focus on the binding of the cofactor, CoB_{12},[1] and its derivatives or various breakdown products (Yamada *et al.*, 1971; Yamada and Hogenkamp, 1972; Tamao and Blakley, 1973), information about the direct interaction of the enzyme with its effectors and substrates has been lacking. This report summarizes results of such studies on the direct binding of effectors and substrates to enzyme.

Experimental Section

Purification of Ribonucleoside Triphosphate Reductase. A recently reported purification procedure for this enzyme (Panagou *et al.*, 1972) indicated that a homogeneous protein was obtained after a rather limited purification. The implication that a relatively large fraction of the extractable protein is ribonucleoside triphosphate reductase is confirmed by the fact that a very different purification scheme that has been used in this laboratory over a period of several years also yields a homogeneous enzyme after a 30-fold purification of a soluble extract obtained from bacteria grown in crude media.

Bacteria. L. leichmannii (ATCC 7630) was grown commercially by Grain Processing Co., Muscatine, Iowa, in 200-gal. fermenter batches using a crude growth medium based upon glucose, yeast extract, protein hydrolysate, and inorganic salts, and containing 0.1% Tween 80. The pH was maintained between 5.5 and 7.0 by periodic additions of 1-l. aliquots of 10 M NaOH. Cells were grown at 37° without agitation under essentially anaerobic conditions, and they were harvested during logarithmic growth when the volume of packed cells reached approximately 1% of the culture volume. For harvest, the culture was cooled and centrifuged, and the packed cells were frozen in paper cartons. No detectable loss of enzyme has been observed during several years of storage of packed bacteria at −65°.

Crude Extract. The reductase was quantitatively released into solution by treating the bacteria in a dilute buffer solution with lysozyme. A cell suspension was prepared by stirring 1400 g of partially thawed bacterial paste with 4200 ml of 0.03 M potassium phosphate buffer (pH 7.2) in a blender (Sorvall Omni-mix) operated for 30 sec at 60 V (in batches of 100 g/300 ml of buffer). Crystalline lysozyme was added to the sus-

† From the Department of Biochemistry, Faculty of Arts and Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15261. *Received September 24, 1973.* Supported in part by Public Health Service Research Grant No. CA-08395 from the National Cancer Institute and by Grant No. E-1M from the American Cancer Society.

[1] Abbreviations used are: CoB_{12}, 5′-deoxyadenosylcobalamin; Dnp, dinitrophenyl; Gdn·HCl, guanidine hydrochloride.

TABLE I: Purification of Ribonucleoside Triphosphate Reductase from Large Batches of *Lactobacillus leichmannii*.[a]

Preparation number:		III		IV		VI	
Packed bacteria (kg):		1.4		1.6		1.6	
Fraction	Vol (ml)	Protein (g)	Enzyme (Units)	Sp Act. (Units/mg)	Enzyme (Units)	Sp Act. (Units/mg)	Enzyme (Units)	Sp Act. (Units/mg)
A (extract)	4560	34.2	18,200	0.53	21,600	0.55	20,000	0.55
B (streptomycin)	5230		18,400		19,000		17,600	
C (ammonium sulfate)	260	10.4	17,000	1.6	18,700	1.6	19,300	1.6
D (pH 5)	150	5.0	16,800	3.4	18,200	3.4	19,500	3.4
E (DEAE-cellulose)	79	1.6	11,800	7.4	8,700	7.1	14,000	7.8
F1 (hydroxylapatite 1)	58	0.96	9,500	9.9	9,000	11.3	10,400	10.8
F2 (hydroxylapatite 2)	54	0.65	8,600	13.2	8,200	16.0	7,000	15.4

[a] The three largest-scale preparations are summarized in terms of enzyme recovery and purification. Enzyme units (μmol of CTP reduced to dCTP per hr) were measured as described in Methods.

pension 1 g/100 g of bacterial paste) which was then rapidly heated to 37°. After 20 min with constant stirring at 37°, the suspension was rapidly chilled in ice to 5°. The heating and cooling periods each occupied 5–10 min. All further steps in the purification procedure were carried out at 0° to 4°. Centrifugation for 20 min at 15,000*g* yielded a faintly opalescent extract (A). The analytical data on this and subsequent fractions are summarized in Table I. Repeated extraction and homogenization of the cell residue yielded less than 10% of the reductase activity of fraction A.

Streptomycin Treatment. Nucleic acids were removed and the extract was clarified further by treating fraction A in bactches of 1000–1500 ml with one-sixth volume of 10% streptomycin sulfate in water. The streptomycin was added at a rate of 10 ml/min with constant stirring, and the stirring was continued for an additional 30 min. Centrifugation for 10 min at 15,000*g* yielded a clear solution (fraction B) with no loss of activity.

Ammonium Sulfate Precipitation. The enzyme was precipitated by first mixing fraction B with an equal volume of 0.5 M potassium phosphate buffer (pH 7.2) and then adding 340 g of solid ammonium sulfate/l. of buffered solution. After 30 min, the precipitate was collected by centrifugation and dissolved in a minimal volume of 0.05 M potassium phosphate buffer (pH 7.2) to yield fraction C. Preparations have been stored frozen for long periods at this stage.

Acid Precipitation. Contaminating proteins were found to be less soluble than the reductase at pH 5.0. Fraction C was diluted with water to a protein concentration of 10–12 mg/ml and dialyzed overnight against distilled water. Any precipitate was removed by centrifugation and discarded, and the protein concentration was adjusted to 5 mg/ml by addition of water. The pH of the solution was lowered to 5.0 by slow addition of 1 M acetic acid. The precipitate was removed by centrifugation and discarded and the supernatant solution was adjusted to pH 7.0 by titration with 1 M NaOH. Finally, the enzyme was precipitated by the addition of ammonium sulfate to 0.8 saturation, and the precipitate was dissolved in a minimal volume of 0.05 M potassium phosphate buffer (pH 7.2) to yield fraction D.

Chromatography on DEAE-cellulose. Fraction D was dialyzed overnight against 0.01 M potassium phosphate buffer (pH 7.2) containing 1 mM β-mercaptoethanol. The dialyzed solution was applied to a column of 500 g of DEAE-cellulose (Whatman DE-52, 5-cm diameter) that had been equilibrated with the same solvent. Inactive protein was removed by elution with 1400 ml of 0.1 M potassium phosphate buffer (pH 7.2) containing 1 mM mercaptoethanol (flow rate, 2 ml/min). Increasing the buffer concentration to 0.15 M resulted in elution, between 600 and 1000 ml, of a protein peak containing the enzyme. The enzyme was again precipitated at 0.8 saturation with ammonium sulfate and dissolved in a minimal volume of buffer to yield fraction E.

Chromatography on Hydroxylapatite. Fraction E was dialyzed overnight against 0.01 M potassium phosphate buffer (pH 6.8) containing 1 mM mercaptoethanol, and it was applied to a hydroxylapatite column (5 × 30 cm) that had been equilibrated with the same solvent. The hydroxylapatite was prepared by a standard method (Tiselius *et al.*, 1956). The enzyme was eluted by increasing the phosphate concentration to 0.075 M, maintaining a flow rate of 1 ml/min, and collecting 6-ml fractions. A yellow inactive protein fraction preceded the enzyme peak with greater or lesser overlap depending upon the batch of hydroxylapatite used. The fractions of highest specific activity eluted between 650 and 1100 ml, and these were combined and precipitated with ammonium sulfate (0.8 saturation). The precipitates were dissolved in a minimal volume of 0.05 M buffer as in the previous steps to yield fraction F1. Attempts to improve the resolution in this step by using elution gradients, other pH's, or other elutants were not successful. Normally, a second consecutive hydroxylapatite fractionation, a duplicate of the first, was carried out with fraction F1 in order to complete the elimination of the early-eluting contaminant. Conditions were the same except that the column length was reduced to 22 cm and protein was eluted between 450 and 850 ml. Fractions of constant specific activity were combined, and they were concentrated by ultrafiltration through Amicon-UM3 membranes to yield fraction F2. It has not been possible to obtain any additional increases in specific activity beyond this step, and the electrophoretic and centrifugal studies reported below indicate that the preparation is essentially homogeneous.

Comparison of the specific activity of the enzyme (see Table I) at the final stages of the purification used here with that reported by others (Panagou *et al.*, 1972) indicates an apparent 8-fold discrepancy. The assay conditions used by us (see below) are quite different from those used in the cited study, however. When activity measurements were made on our enzyme according to the conditions used by Panagou *et al.*,

the specific activity was within experimental error of that reported earlier.[2]

Methods

Enzyme Assay. Ribonucleoside triphosphate reductase activity was routinely measured by incubating enzyme fractions (approximately 0.1 unit) for 10 min at 37° in 0.20 ml of 0.05 M histidine buffer (pH 7.0) containing 0.07 M dithiothreitol, 10 mM $MgCl_2$, 0.125 mM dATP, 10 μM CoB_{12}, and 0.5 mM [^{3}H]CTP. Incubations, carried out in the dark, were terminated by chilling to 0°, followed by addition of 2.5 μmol of unlabeled dCMP carrier and perchloric acid to a final concentration of 0.5 M. After hydrolysis (15 min at 100°) to convert dCTP to dCMP, and the removal of perchlorate by precipitation with KOH, the solutions were adjusted to 0.2 M acetic acid and chromatographed with the same solvent on Dowex 50 columns (1 × 13 cm) designed to automatically collect the dCMP fraction eluted between 190 and 260 ml (Turner *et al.*, 1966). Aliquots were assayed for ^{3}H and for absorbance at 280 nm, and the yields of [^{3}H]dCTP were calculated. The amount of dCTP formed was routinely found to be a linear function of time, and the reaction rate was linearly related to enzyme concentration. A unit of enzyme activity is defined as the amount catalyzing reduction of 1 μmol of CTP/hr under the foregoing conditions. Protein concentrations were determined by the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as a standard.

Ultracentrifugation. Measurements of molecular weight by the meniscus depletion method of equilibrium centrifugation (Yphantis, 1964), sedimentation velocity, and diffusion were carried out with the Beckman-Spinco Model E analytical ultracentrifuge. The diffusion coefficient was determined at low rotor speed by the analysis of a synthetically formed boundary. The concentration dependence of the sedimentation coefficient was evaluated by a differential sedimentation method akin to that of Schumaker and Adams (1968). A value of 0.73 $cm^3\ g^{-1}$ was chosen for the partial specific volume of the native protein based upon the reported amino acid composition (Panagou *et al.*, 1972). For denatured enzyme in solutions containing 6 M Gdn·HCl, an apparent specific volume of 0.72 $cm^3\ g^{-1}$ was adopted (Noelken and Timasheff, 1967; Hade and Tanford, 1967). The centrifugations in 6 M Gdn·HCl were carried out with exhaustively dialyzed samples, and the dialysates were used in the reference channel of the double-sector centerpiece. For the sample of maleylated enzyme, the value of the native protein partial specific volume was used. Equilibrium centrifugations in dilute buffer solutions were carried out for at least 20 hr. Those in concentrated Gdn·HCl solutions ran for 48 hr and longer. The attainment of equilibrium was generally checked by comparing results from centrifugations extending over several time spans.

Gel Electrophoresis in Sodium Dodecyl Sulfate. Polyacrylamide gels with 5% and 7.5% cross-linkage were prepared in the presence of 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulfate for the evaluation of molecular weight (Shapiro *et al.*, 1967). Ribonucleoside triphosphate reductase and reference solution (hemoglobin, trypsin, pepsin, ovalbumin, bovine serum albumin, and γ-globulin) were prepared by dissolving 400 μg of each in 0.25 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 1% sodium dodecyl sulfate and 1% mercaptoethanol, followed by incubation at 37° for 3 hr. An aliquot of the stock ribonucleoside triphosphate reductase solution was similarly denatured and reduced although 0.1 M sodium borate buffer (pH 9.0) was used in place of phosphate. After the incubation period, this protein was treated with excess iodoacetate. Some samples of enzyme were heated in the sodium dodecyl sulfate–mercaptoethanol solution for 40 min at 60° to accelerate denaturation and separation of any possible subunits. One aliquot of γ-globulin was processed in the same manner as the other marker proteins but with the omission of mercaptoethanol at all stages, including dialysis. The treated samples were dialyzed against 0.01 M phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulfate and 0.1% mercaptoethanol. Sucrose was added to the samples to a concentration of approximately 10% and aliquots of 50–100 μg were layered under the electrode buffer on the 10-cm gel columns. Electrophoresis was carried out for 4 hr at 2.5 mA/gel tube, after which the gels were fixed and stained with Coomassie Blue. Mobility was measured relative to a marker dye, Bromophenol Blue.

Gel Filtration in Sodium Dodecyl Sulfate. Molecular size determinations by filtration of denatured proteins on columns of Sephadex G-200 (Pharmacia) were made as follows. Samples of the reference proteins, trypsin, ovalbumin, and bovine serum albumin, and of the reductase (2 mg each) were incubated at 37° for 4 hr in 0.4 ml of 0.5 M sodium dodecyl sulfate containing 4 M urea. In some cases 0.1 M dithiothreitol or 0.1 M mercaptoethanol were present in the reductase incubation mixture. These samples were alkylated by incubation for 1 hr with a 5-fold excess of iodoacetamide with the pH maintained at 7–8 by the addition of NaOH. All samples were dialyzed against 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M sodium dodecyl sulfate and 0.02 M iodoacetamide, and finally against identical solvent from which iodoacetamide was omitted. The samples were applied individually to a 1 × 80 cm column of Sephadex G-200 that had previously been equilibrated with the same buffer–sodium dodecyl sulfate solution. The same solvent mixture was used to wash samples through the column at a rate of 0.75 ml/min while the effluent solution was monitored for protein by 280-nm absorbance. Samples of dextran blue (Pharmacia) were used to measure the void volumes of the columns. Mobility was characterized in terms of the ratio of the difference in elution and void volumes to the void volume.

Gel Filtration in Agarose. With 6 M Gdn·HCl as a denaturing solvent, molecular sizes were determined by filtration on 1 × 90 cm columns of agarose (Bio-Gel A-5M) using a modification of the procedure of Davison (1968). Samples of 2 mg each of reductase, hemoglobin, trypsin, ovalbumin, and bovine serum albumin were dissolved in 0.4 ml of 6 M Gdn·HCl containing 0.05 M dithiothreitol, 0.01 M EDTA, and 0.05 M LiCl, adjusted to pH 7.2. Protein samples, together with dextran blue and Dnp-methionine as markers to determine the void and internal volumes, were applied to columns that had been equilibrated with the Gdn·HCl solvent mixture, and the same solvent was also used to wash the samples through the columns at a flow rate of 2 ml/hr. The protein in the eluate was measured turbidimetrically at 440 nm after addition of trichloroacetic acid. Mobility was characterized in terms of the ratio of the difference in elution and void volumes to the internal volume.

Equilibrium Dialysis Measurements. Except when otherwise stated nucleoside phosphates for binding studies were obtained from Schwarz BioResearch and used without further purification. Radiolabeled materials had specific activities of 5–

[2] The comparative assays of ribonucleotide reductase activity by the procedure of Blakley and his collaborators and by the procedure used in this study were performed by Dr. Malcolm D. Orr in our laboratories.

50×10^5 cpm/μmol. The amounts of impurities in the form of nucleoside mono- and diphosphates were determined by thin-layer chromatography employing MN-300 cellulose on plastic support sheets distributed by Brinkmann Instruments, Inc. (Randerath and Randerath, 1967). Appropriate corrections were made to the equilibrium dialysis data based on the assumption, consistent with kinetic studies, that the mono- and diphosphate forms of the nucleotides do not bind to the enzyme. Equilibrium dialysis was carried out essentially according to the procedure of Englund *et al.*, with cells having the same chamber dimensions but of modified design (Englund *et al.*, 1969). The cells were assembled with 1-cm square pieces of stretched Union Carbide 20/32 dialysis tubing and with two 1-mm glass beads in each chamber to aid mixing. The same volume, 20 μl, of protein and radiolabeled ligand solutions were loaded into the membrane-separated chambers of the assembled cell with a 25-μl Hamilton syringe having a needle of reduced outside diameter. The cells were sealed with tape and mounted on a vertical disk revolving at 6 rpm. After attainment of equilibrium, 90 min, duplicate 4- or 5-μl aliquots were withdrawn from each cell chamber with a dry Hamilton syringe and transferred to counting vials. Scintillation fluid, 10 ml, comprised of 2 ml of Triton X-100, 8 ml of toluene and 32 mg of Omnifluor (New England Nuclear), was then added to each vial and the level of radioactivity was determined by scintillation counting.

Protein concentrations were in the range of 2–8 mg/ml and ligand concentrations, for direct binding studies, ranged from 10 to 2000 μM. Potassium phosphate (0.05 M, pH 7) was routinely used as the buffered solvent in all ligand-binding studies. In contrast to the observations of Beck, the enzyme is only mildly responsive to Mg^{2+} ion levels (Beck, 1967). Since the absence of Mg^{2+} ion from the assay medium has relatively little effect on the catalytic activity of the enzyme, it was routinely omitted from binding studies except where indicated later. Dialyses were performed at 22–23°. Assays of the protein containing solution before and after equilibrium dialysis showed decreases in enzymatic activity of not more than 20%. Thin-layer chromatography of nucleoside triphosphate solutions used for equilibrium dialysis showed no detectable amount hydrolyzed. Recovery of the added isotopic label was always quantitative, indicating that these ligands do not adhere to the partitioning membrane or the cell. In a few cases ligand was added initially only to the protein side of the dialysis cell. The same degree of binding was obtained as with the normal loading protocol demonstrating the reversibility of the binding processes and the validity of thermodynamic analysis. Calculations of binding ratios were based on the molecular weight of the protein being 76,000 daltons.

Differential Fluorescence Measurements. Binding ratios were determined by fluorescence measurements by a method which self-compensates for optical inner filter effects (Franzen *et al.*, 1972). The amount of quenching of the protein fluorescence due to ligand binding was measured at 23° with an Aminco-Bowman 4-8100 spectrophotofluorometer with excitation at 285 nm and emission at 330 nm. For each type of deoxynucleoside triphosphate, used as a ligand for a binding study, the corresponding deoxynucleoside or deoxynucleoside monophosphate was used as a pseudoligand for the reference cuvet in accord with the protocol of the differential fluorescence method (Franzen *et al.*, 1972). Initial protein concentrations ranged from 0.3 to 1.0 mg per ml, the total volume of solution being 1.2 ml. For each experiment about 15 successive portions of stock ligand (or pseudoligand) solution were added, the added volume accumulating to about 0.38 ml.

Results

Molecular Parameters. Equilibrium molecular weight determinations as summarized in Tables II and III are in accord with those reported by Panagou *et al.* (1972). The molecular weight of 75,300 observed for the native enzyme was essentially unaffected by a variety of conditions designed to separate subunits if they existed. It is noteworthy that incubation of the enzyme at 60° in 6 M Gdn·HCl for 20 min did not lead to any reduction in molecular weight. Furthermore, the introduction of excess charge by maleylation did not lead to dissociation.

TABLE II: Physical Parameters of Native Ribonucleoside Triphosphate Reductase.

Parameter and Conditions	Value
$A_{280}^{1\%}$, 1 cm (based upon Kjeldahl N analysis)	12.0
$s^0_{20,w}$ (sec)	5.13×10^{-13}
ds/dc (sec cm^3 mg^{-1})	-0.057×10^{-13}
$D_{20,w}$ (cm^2 sec^{-1})	5.92×10^{-7}
Axial ratio (for prolate ellipsoid with a solvation factor of 0.2 g/g, determined from D)	3.6
Molecular weight (by s/D)	7.84×10^4
Molecular weight (by Yphantis equilibrium method)	7.53×10^4

TABLE III: Molecular Weight of Ribonucleoside Triphosphate Reductase as Determined by Equilibrium Ultracentrifugation under Various Denaturing Conditions.

State of Enzyme Preparation	Mol Wt
Native enzyme in 0.05 M phosphate (pH 7.0)	75,300
Enzyme in 0.1 M glycine buffer (pH 9.1)	72,500
Enzyme in 6 M Gdn·HCl–0.1 M dithiothreitol	69,300
Enzyme in 6 M Gdn·HCl–0.1 M dithiothreitol, heated for 20 min at 60° prior to centrifugation	74,500
Maleylated enzyme in 0.05 M phosphate buffer after reduction with mercaptoethanol and treatment with maleic anhydride (Sia and Horecker, 1968)	72,600

Similarly, electrophoretic and gel filtration studies, performed under a parallel set of denaturing and subunit dissociating conditions and summarized in Figure 1, repeatedly indicated a functional mobility, for ribonucleoside triphosphate reductase corresponding to a polypeptide chain molecular weight of 75,000–80,000 daltons. Again we note that preheating at 60°, in this case in 0.1% sodium dodecyl sulfate plus reducing agent, caused no alteration in electrophoretic mobility. All of these attempts to measure molecular size under denaturing conditions indicate that the native enzyme has no quaternary structure, a conclusion reached independently by Panagou *et al.* (1972).

It could be argued that the enzyme dimerizes in the presence of all the reagents needed for ribonucleotide reduction, and that the characteristics of allosterism result from the quaternary struc-

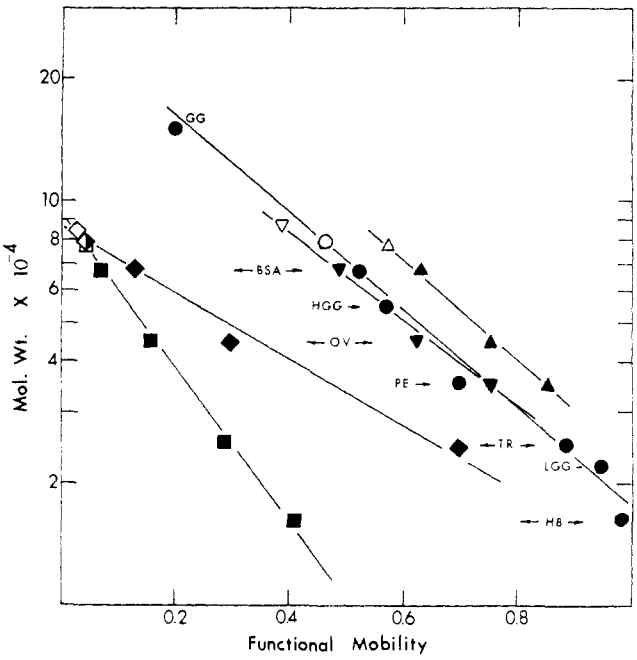

FIGURE 1: Molecular weight estimations from sodium dodecyl sulfate gel electrophoresis and gel filtration. Proteins were treated and eluted as described in Methods. The reference proteins were GG, γ-globulin; BSA, bovine serum albumin; HGG, heavy chains of γ-globulin; OV, ovalbumin; PE, pepsin; TR, trypsin; LGG, light chains of γ-globulin; HB, hemoglobulin subunits. Open symbols represent ribonucleotide reductase migration. Electrophoretic mobility in cross-linked polyacrylamide gel containing 0.1% sodium dodecyl sulfate: (○) reductase incubated with sodium dodecyl sulfate–mercaptoethanol for 3 hr at 37° prior to electrophoresis in 5% cross-linked gel; (△) reductase subjected to additional incubation in sodium dodecyl sulfate–mercaptoethanol for 40 min at 60° prior to electrophoresis; (▽) electrophoresis of heated reductase in 7.5% cross-linked gel. Chromatographic mobility: (◇) chromatography on Sephadex G-200 eluted with 0.1 M sodium dodecyl sulfate–0.01 M phosphate buffer (pH 7.0) (the half-shaded symbol represents alkylated reductase); (□) chromatography on agarose eluted with 6 M Gdn·HCl–0.05 M dithiothreitol.

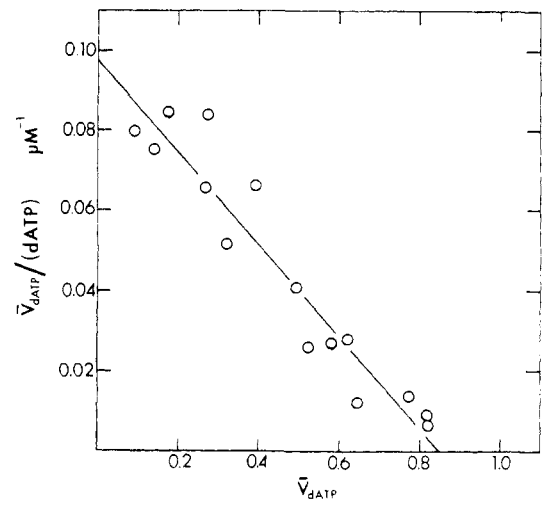

FIGURE 2: Scatchard plot of dATP binding to ribonucleotide reductase.

ture of the active enzyme. We have not determined the molecular weight of the enzyme in the presence of the assay medium components. We have, however, examined the differential sedimentation of the enzyme in presence and absence of all reactants except reducing agent by the method of Schumaker and Adams (1968). The sedimentation of ribonucleoside triphosphate reductase in 0.05 M phosphate buffer at pH 7 was not distinguishably affected by the copresence of added $MgCl_2$, dATP, CTP, and CoB_{12}, at final molar concentrations of 3×10^{-3}, 3.2×10^{-4}, 6.6×10^{-4}, and 1.5×10^{-4}, respectively. Moreover, the sedimentation rate of the enzyme in a sucrose gradient was unaltered by the added presence of all reaction components at uniform concentration throughout the gradient. We conclude that the enzyme remains monomeric when it engages in catalysis, and, therefore, any cooperative intersite effects must take place within one polypeptide chain.

Effector Binding. In Table IV are shown the data from a representative direct binding experiment by the equilibrium dialysis technique using dATP as the ligand. Values from this table, along with those obtained from other companion experiments are presented in Figure 2 in the form of a Scatchard plot. From the linear relationship over a wide range of effector concentration, it is determined that only one dATP effector molecule was bound to the enzyme, forming a moderately stable binary complex characterized by a dissociation constant of 8.7 μM. Similar linear relationships were observed with other deoxynucleoside triphosphate effectors. These binding data are summarized in Table V. The dissociation constants determined by two different methods show reasonably good agreement. The entries for dATP binding in the last two columns demonstrate the absence of any pronounced effect of ionic strength on these binary complex stabilities in the range of ionic strength examined.

To ascertain whether or not each of the effector molecules was binding to one and the same effector site, competition experiments were performed in which increasing amounts of unlabeled effector, B, were added to a series of dialysis cells, each containing a fixed amount of radiolabeled effector of another type, A. It is readily shown that for this one-site two-ligand situation, the following relationship pertains between $\bar{\nu}_A$, [A] and [B], where $\bar{\nu}_A$ is the binding ratio of radiolabeled ligand, K_A and K_B are dissociation constants, and [A] and [B] are the molar concentrations of each ligand (Changeux *et al.*, 1968).

$$[A][(1 - \bar{\nu}_A)/\bar{\nu}_A] = K_A + (K_A/K_B)[B]$$

The results of the binding studies carried out in this manner are summarized in Table VI, and a specific example illustrating the functional form of the release of initially bound ligand by addition of competitor is shown in Figure 3. From these data it is evident that the effectors do vie for the same site on the enzyme regardless of the chemical nature of the base, be it purine or pyrimidine. The dissociation constants of Table VI show the same relative magnitudes as those obtained by direct binding studies (see Table V); however, the absolute magnitudes are somewhat higher. The origin of this discrepancy is not entirely certain.

It should be noted that the concentrations of competitors actually used to generate the plots of Figure 3 and similar plots were obtained by dividing the moles of competitor added by the total volume of solution in the dialysis cell. Since some of the added competitor is protein bound, the concentration of free competitor should really be less than that shown, for example, in Figure 3. The estimated correction for the ligand combination wherein the error is most serious, namely, dGTP–dATP, leads to a 10% decrease in the dGTP dissociation constant reported in Table VI. For the other deoxy-

TABLE IV: Ribonucleotide Reductase Binding of dATP by Equilibrium Dialysis.

Cell No.	Radioact of Chambers[a] at Equil (cpm/4 μl)			dATP Concn (μM)		Binding Ratio, $\bar{\nu}$ (mole of dATP/ mole of Protein)
	Solvent (Obsd)	Chamber (Cor)[b]	Protein Chamber	Free	Bound	
1	26.2	22.8	108.3	1.17	4.22	0.0929
2	47.8	41.4	206.6	2.13	8.16	0.180
3	74.7	64.8	321.4	3.33	12.7	0.278
4	132.8	117.4	485.1	6.03	18.1	0.398
5	263.0	238.8	703.7	12.3	22.6	0.497
6	459.6	423.7	977.2	21.8	26.6	0.585
7	816.4	762	1372	39.1	28.6	0.629
8	1214	1137	1902	58.4	35.3	0.778
9	1957	1841	2688	94.5	37.6	0.826
10	2763	2606	3494	133.8	37.6	0.828

[a] Each chamber contained 20-μl total volume. Buffer composition and other experimental details are described in the equilibrium dialysis section of Methods. The concentration of protein was 45.5 μM. The specific radioactivity of the added ligand was 4868 cpm/mmol after correction for the presence of 3% impurity of nonradiolabeled dAMP and dADP and 5% radiolabeled impurity. [b] This correction is based on the assumptions that the radiolabeled impurity does not bind to the enzyme and that it is freely diffusible. Hence, $cpm_{cor} = cpm_{obsd} - (1/2)0.05\ cpm_{added} = cpm_{obsd} - 0.025(cpm_{in} + cpm_{out})_{obsd}$.

TABLE V: Deoxyribonucleoside Triphosphate Binding by Ribonucleotide Reductase.

Ligand	Equilibrium Dialysis		Differential Fluorescence[a]	
	No. of Sites	Dissoc Constant[b] (μM)	Dissoc Constant[b] (μM)	Ionic Strength
dATP	0.85 ± 0.04	8.7 ± 0.8	12.0 ± 1.3	0.02
			12.8 ± 1.5	0.02[c]
			8.9 ± 1.1	0.10[c]
dGTP	1.03 ± 0.05	16.4 ± 0.8	12.2 ± 1.5	0.02
dCTP	1.02 ± 0.02	83 ± 7	48 ± 5	0.02
dTTP	1.19 ± 0.07	55 ± 6	50 ± 5	0.02

[a] This method yields dissociation constants calculated on the assumption that only binary complexes are formed. [b] Reported standard deviations are those of the slopes of unweighted linear least-squares analysis of Scatchard plots. [c] These two entries refer to experiments done in 0.005 M phosphate buffer at pH 7, the ionic strength being varied by the addition of KCl. All other determinations were made in 0.05 M phosphate buffer at pH 7.

TABLE VI: Dissociation Constants Obtained by Competition Studies with dATP as the Radiolabeled Primary Ligand.

Ligand	K_{dATP} (μM)	K_B (μM)
dGTP	22.6 ± 5.0	33.3 ± 2.9
dCTP	35.0 ± 4.2	185 ± 14
dTTP	26.9 ± 0.5	100 ± 6
dITP	36.9 ± 2.0	204 ± 13
ATP	29.8 ± 3.4	7,050 ± 360
GTP	27.4 ± 4.7	14,200 ± 1800
CTP	41.2 ± 4.9	14,000 ± 1200
ITP	84 ● 4	27,000 ± 3000
UTP	Showed no competition	
dCDP	38.2 ± 2.7	2,680 ± 60
AraATP	40.2 ± 1.4	2,580 ± 120

nucleoside triphosphates the difference is smaller and for the substrates which compete with dATP, the correction is negligible. The general conclusions to be drawn from these competition experiments are not jeopardized, therefore, by neglect of this correction.

Many dialysis experiments were performed with the substrates as ligands in the absence of other reaction components, and in the presence of all but one of any of the other reaction components. In these experiments Mg^{2+} ion was sometimes present. Within the limits of detectability using the equilibrium dialysis technique, essentially no substrate binding was manifest in direct binding experiments, even at concentrations as high as a few millimolar. One possible mechanistic model of the catalytic process in the absence of effector casts the substrate in the role of acting as an effector by first binding weakly to the effector site, whereupon the catalytic site becomes more avid for substrate. The inherent attractiveness of this model and the structural similarity between the substrates of enzyme and the known effectors, prompted us to undertake competition binding studies in which substrates served as competitors for the effector site bound by radiolabeled deoxyribonucleoside triphosphates. Indeed, competition was demonstrable in these instances, except for the case of UTP, as can be seen from Figure 3 and Table VI. The values of the dissociation constants of the effector site–substrate binary complexes are of such high magnitude that one would not have expected to observe binding by direct measurement with the available methods. Reference to Figure 3 also shows that the major release of dATP from the enzyme by added CTP is not due to a general medium effect such as enhanced ionic strength, since CDP at similar concentrations did not produce the kind of reduction in dATP binding caused by CTP. The same ineffectiveness as a competitor was observed with ADP as well. That there is some minor general medium effect is probable, however, in light of the repeated observation that the intercepts of plots like those in Figure 3B lie above the ordinate values obtained experimentally in the absence of substrate competitor. This is not the case for competition between

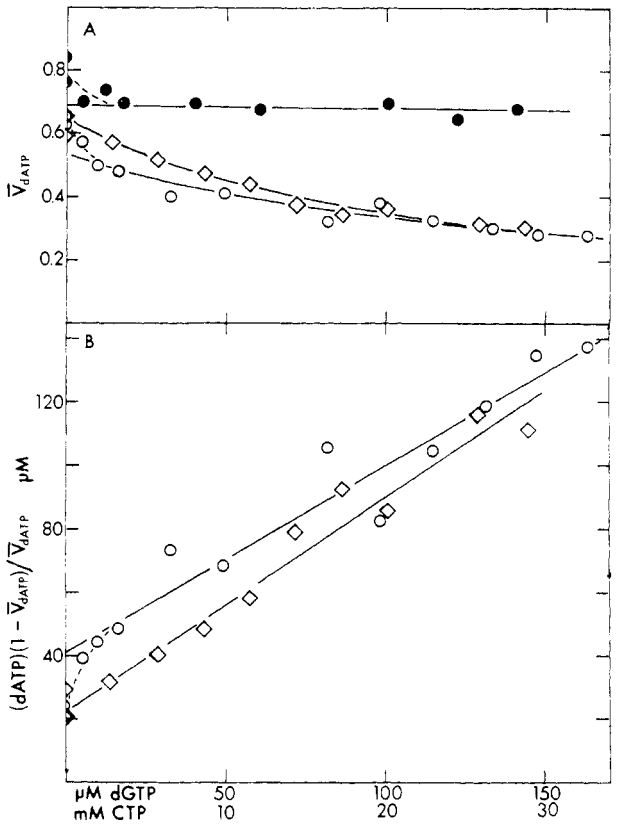

FIGURE 3: dGTP and CTP competition for the effector site occupied by dATP. For the particular experiments summarized in this figure, the reductase concentration was 50 μM and the dATP concentration in the absence of competitor was 40 μM. (A) Effect of dATP binding by added dGTP (◇), CTP (○), and CDP (●). Solid curves through the dGTP and CTP data were generated from the appropriate disociation constants obtained from the slopes and intercepts of the lines in part B. Dashed curves indicate apparent extrapolations to zero competitor concentrations. (B) Linearized form of competition binding data, dGTP (◇) and CTP (○). The dissociation constants obtained from the least-squares lines calculated from the points obtained with competitor present are K_{dATP} = 22.6 ± 5.0 μM, K_{dGTP} = 33.3 ± 2.9 μM (from diamonds), and K_{dATP} = 41.2 ± 4.9 μM, K_{CTP} = 14.0 ± 1.2 mM (from circles).

effectors. Notice, however, that the concentration range over which substrate competes for the effector site is much higher than that for effector *vs.* effector. We propose that between zero and a few millimolar concentration of added substrate there is a medium-induced minor increase in the dissociation constant of the enzyme–effector complex. This effect accompanies true competition.

The interpretation of the observed decrease in dATP binding in the presence of added nucleoside in terms of substrate competition for the effector site presupposes that the nucleoside triphosphates are not contaminated with deoxynucleoside triphosphates. Contamination on the order of a per cent of dCTP would lead to results like those shown in Figure 3A for the CTP–dATP experiment. Assay of CTP by hydrolysis to CMP and ion-exchange chromatography with added radiolabeled dCMP showed that less than 0.01% dCMP was present in the commercial CTP used. It is assumed that the other nucleoside triphosphates were equally pure.

Discussion

Nonkinetic evidence suggestive of binding of the deoxyribonucleoside triphosphate effectors to ribonucleoside triphosphate reductase has been reported. The electron spin resonance spectra of enzyme bound cob(II)alamin, for example, show definite unique responses to the presence of either effectors or substrates (Hamilton *et al.*, 1971). Similarly, electron paramagnetic resonance spectra of an apparent active enzyme-bound cobamide intermediate which temporally precedes cob(II)alamin, display features that are sensitive to the nature of the effector and substrate or substrate analogs present (Hamilton *et al.*, 1972). It has also been shown by ultrafiltration measurements that ATP and dGTP substantially enhance the association of the CoB_{12} component, 5′-deoxyadenosine, to the enzyme, presumably by binding to a neighboring site (Yamada *et al.*, 1971). Finally, the requirement of the presence of effector for the adsorption of the enzyme by affinity chromatography with a 5′-deoxyadenosylcobalamin–agarose matrix also argues for effector binding by the enzyme (Yamada and Hogenkamp, 1972). This report summarizes attempts to measure directly the stability of enzyme–effector or enzyme–substrate complexes under equilibrium conditions, and to determine the stoichiometry of enzyme–effector or enzyme–substrate complexes. The results clearly demonstrate the existence of enzyme–effector binary complexes in the absence of other reaction components at concentrations of effector comparable to those required to influence substrate specificity in the overall reactions (Vitols *et al.*, 1967b). Clearly, the enzyme is univalent for effector, and one and the same site appears to serve for all effectors. This fact raises some doubt about the interpretation of nucleoside triphosphate effects on the hydrogen exchange between deoxyadenosylcobalamin-5′-t_2 and water (Follman and Hogenkamp, 1971). Based on differential effects of various nucleotides on substrate reduction and tritium exchange these authors proposed the existence of two effector sites, each functioning to modulate one of the two activities. Under no conditions, including the presence of reducing agent, have we observed the binding of more than one effector molecule per protein molecule.

It is interesting that we fail to observe any affinity between enzyme and substrate at other than the effector site. By inference from the electron spin resonance studies on complete reaction systems and the lack of directly observed substrate binding in the necessarily incomplete systems investigated in equilibrium-binding experiments reported here, we suggest that the ribonucleotide substrate must be the last entity to interact with the enzyme.

It is probable that the enzyme itself is, in some incompletely understood manner, primed for ribonucleotide substrate reduction by dithiols (Vitols *et al.*, 1967b; Tamao and Blakely, 1973). Dithiols alone, however, do not alter the enzyme for ribonucleotide binding to a substrate site, since even in the presence of dithiothreitol we could detect no such binding by equilibrium dialysis. This observation is consistent with the view that substrate is the last entry in an ordered reaction sequence for the enzyme.

Reduction of ribonucleotides does occur at measurable, though somewhat reduced, rates in the absence of deoxyribonucleotide effectors (Goulian and Beck, 1966; Beck, 1967; Vitols *et al.*, 1967a). Our own kinetics studies, employing the assay described in this paper, indicate that half-maximal velocity of CTP reduction is reached at about 10^{-3} M in the absence of dATP and at about 10^{-4} M in the presence of dATP. Even allowing for the possibility that ribonucleotide substrate is the last reactant to adhere to the enzyme, one is prompted to ask whether the observed weak binding of substrate to the effector site ($K \simeq 10^{-2}$ M) can be consistent with the finding that half-maximal velocity is achieved at substrate

concentrations of 10^{-3} M in the absence of effectors. It is possible to rationalize these facts with a simplistic model such as the following. Let us suppose that substrate can bind both to the effector site and to the catalytic site. We shall assume that the dissociation constant for the substrate–catalytic site complex is invariant having a dissociation constant equivalent to the substrate half-saturation concentration with effector present, approximately 10^{-4} M. This site, however, shall be presumed to exist only if all other reaction components are present, and if the effector site is occupied either with effector or substrate. We shall further assume that the effector site binding is characterized by the dissociation constant values listed in Tables V and VI. It is easily shown that such a model leads to a catalytic site saturation function, f, given by

$$f = \left[1 + \frac{K_{sub}}{[S]}\left(1 + \frac{K_{eff}}{[S]}\right)\right]^{-1}$$

Choosing K_{eff} for CTP from Table VI as approximately 10^{-2} M and assuming as noted above that K_{sub} is 10^{-4} M, we find that half-saturation is achieved at a CTP concentration of 10^{-3} M, in keeping with kinetic observations in the absence of effector. Hence, the weak affinity of the substrates for the effector site is not inconsistent with kinetic data on the enzyme obtained in the absence of added deoxyribonucleotides.

A few final comments about some of the features of the ligand molecule which are requisite for binding can be made in consideration of Table VI. Reference to the progression in dissociation constants for the cytosine nucleotide series

$$dCTP \ll dCDP < CTP \ll CDP$$

shows that loss of the terminal phosphate from dCTP greatly enhances dissociation, but even greater loss of affinity results from replacing one of the hydrogens on 2-C′ of dCTP with a hydroxyl group in the ribo configuration. If both of these alterations are made on dCTP, virtually no binding occurs. Similarly, the adenine nucleotide series demonstrates this conclosion, if one considers the following relationships for dissociation constants. The deoxy condition at the 2-C′ position and the presence

$$dATP \ll araATP < ATP \ll ADP$$

of the tertiary phosphate are essential for tight binding at the effector site. Alteration at either location greatly weakens but does not destroy affinity. The nature of the base influences binding significantly, but not as drastically. The purine nucleotides, dATP and dGTP, bind more firmly than the pyrimidine nucleotides, dTTP and dCTP. These latter effects presumably are important in the fine control of the relative rates of nucleotide reduction in the cell.

References

Beck, W. S. (1967), *J. Biol. Chem. 242*, 3148.

Changeux, J.-P., Gerhart, J. C., and Schachman, H. K. (1968), *Biochemistry 7*, 531.

Davison, P. F. (1968), *Science 161*, 906.

Englund, P. T., Huberman, J. A., Jovin, T. M., and Kornberg, A. (1969), *J. Biol. Chem. 244*, 3038.

Fisher, J. R. (1972), *Arch. Biochem. Biophys. 152*, 638.

Follman, H., and Hogenkamp, H. P. C. (1971), *Biochemistry 10*, 186.

Franzen, J. S., Kuo, I., and Chung, A. E. (1972), *Anal. Biochem. 47*, 426.

Goulian, M., and Beck, W. S. (1966), *J. Biol. Chem. 241*, 4233.

Hade, E. P. K., and Tanford, C. (1967), *J. Amer. Chem. Soc. 89*, 5034.

Hamilton, J. A., Tamao, Y., Blakley, R. L., and Coffman, R. E. (1972), *Biochemistry 11*, 4696.

Hamilton, J. A., Yamada, R., Blakley, R. L., Hogenkamp, H. P. C., Looney, F. D., and Winfield, M. E. (1971), *Biochemistry 10*, 347.

Lowry, O. H., Rosebrough, N. J., Farr, L. A., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Noelken, M. E., and Timasheff, S. N. (1967), *J. Biol. Chem. 242*, 6080.

Panagou, D., Orr, M. D., Dunstone, J. R., and Blakley, R. L. (1972), *Biochemistry 11*, 2378.

Randerath, K., and Randerath, G. (1967), *Methods Enzymol. 11*, 461.

Schumaker, V., and Adams, P. (1968), *Biochemistry 7*, 3422.

Shapiro, A. L., Viñuela, E., and Maizel, J. V., Jr. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.

Sia, C. L., and Horecker, B. L. (1968), *Biochem. Biophys. Res. Commun. 31*, 731.

Tamao, Y., and Blakley, R. L. (1973), *Biochemistry 12*, 24.

Tiselius, A., Hjerten, S., and Levin, O. (1956), *Arch. Biochem. Biophys. 65*, 132.

Turner, M. K., Abrams, R., and Lieberman, I. (1966), *J. Biol. Chem. 241*, 5777.

Vitols, E., Brownson, C., Gardiner, W., and Blakley, R. L. (1967a), *J. Biol. Chem. 242*, 3035.

Vitols, E., Hogenkamp, H. P. C., Brownson, C., Blakley, R. L., and Connellan, J. (1967b), *Biochem. J. 104*, 58C.

Yamada, R., and Hogenkamp, H. P. C. (1972), *J. Biol. Chem. 247*, 6266.

Yamada, R., Tamao, Y., and Blakley, R. L. (1971), *Biochemistry 10*, 3959.

Yphantis, D. A. (1964), *Biochemistry 3*, 297.