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Ribonucleotide Reductase from *Escherichia coli.* Identification of Allosteric Effector Sites by Chromatography on Immobilized Effectors†

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ABSTRACT: Ribonucleotide reductase is responsible for the production of deoxyribonucleotides by catalyzing the reduction of ribonucleoside diphosphates. The enzyme is allosterically regulated in a complex way by the nucleoside triphosphates, ATP, dTTP, dGTP, dCTP, and dATP. Ribonucleotide reductase consists of two nonidentical subunits, proteins B1 and B2. Both substrates and allosteric effectors bind exclusively to B1. Binding of protein B1 to dTTP or dATP covalently coupled to Sepharose and elution with concentration gradients of the different nucleoside triphosphate effectors gave information about (1) the arrangement of the effector binding sites on protein B1 and (2) the affinity of the effectors for these sites. Protein B1 thus has two classes of effector binding sites. One class binds all effectors, as demonstrated by elution of the protein from dTTP-Sepharose with dATP, dGTP, ATP, or dCTP. The second class binds only dATP or ATP, since dATP and ATP were the only nucleotides which eluted protein B1 from dATP-Sepharose. These results confirm earlier data obtained by dialysis binding experiments. The eluting concentrations obtained for the different nucleoside triphosphates in experiments with dTTP-Sepharose could be used to calculate unknown dissociation constants for protein B1-effector binary complexes. This was possible, since a plot of the eluting concentrations vs. known dissociation constants was linear.

Ribonucleotide reductase from *Escherichia coli* (EC 1.17.4.1) is an allosteric enzyme which catalyzes the reduction of ribonucleoside diphosphates to the corresponding deoxyribonucleotides (Reichard, 1968). The same enzyme reduces all four common ribonucleoside diphosphates and the allosteric effectors are nucleoside triphosphates.

The allosteric regulation controls both the general activity and the substrate specificity of the enzyme (Larsson and Reichard, 1966a,b). Thus ATP and low concentrations (~1 μM) of dATP stimulate the reduction of CDP and UDP, while dGTP stimulates the reduction of ADP and GDP. Reduction of all four substrates is stimulated by dTTP. High concentrations of dATP (>10 μM) inhibit the enzyme.

Ribonucleotide reductase consists of two nonidentical subunits, protein B1 and protein B2. The separated subunits are inactive, but recombine in the presence of Mg^{2+} ions to form a 1:1 complex, the active enzyme molecule (Thelander, 1973). Protein B2 contains iron and an organic free radical essential for activity (Brown et al., 1969b; Ehrenberg and Reichard, 1972). Protein B1 binds substrates and allosteric effectors, as demonstrated by binding experiments with dialysis techniques (von Döbeln and Reichard, 1976; Brown and Reichard, 1969). In addition, B1 contains redox active dithiols, which supply the electrons necessary for the reduction (Thelander, 1974).

This paper describes the characterization of the allosteric effector binding sites on protein B1 by affinity chromatography on Sepharose columns containing covalently coupled dTTP or dATP.

Experimental Procedure

Materials. Unlabeled nucleotides (at least 97% pure) were obtained from Calbiochem AG or Sigma and tritium-labeled nucleotides were from Amersham. They were used without further purification. α-^{32}P-labeled dGTP and β-^{32}P-labeled ATP were synthesized in this laboratory (Pigiet et al., 1974). Protein B1 (sp act. 400-450 units/mg) was a gift from Dr. Britt-Marie Sjöberg and had been prepared from a λ-lysogenic *Escherichia coli* strain overproducing ribonucleotide reductase (Eriksson et al., 1977). Two different preparations, which were 80-90% pure as determined by gel electrophoresis in the presence of sodium dodecyl sulfate, were used.

Sepharose 4B with covalently coupled dTTP or dATP was kindly provided by Dr. Lars Thelander and had been synthesized according to the method described by Berglund and Eckstein (1972). This involved synthesis of the *p*-aminophenyl esters of the γ-phosphate of dTTP or dATP and coupling of these derivatives to Sepharose via the amino group. The *p*-aminophenyl esters of the nucleoside triphosphates had allosteric effects similar to those of the corresponding unmodified

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nucleotides (L. Thelander, personal communication). The Sepharose contained 0.2 μmol of deoxythymidine nucleotide or 0.3 μmol of deoxyadenosine nucleotide per mL of packed wet gel.

Affinity Chromatography. Affinity chromatography was carried out at 4 °C. Columns containing 0.5 mL of packed wet dTTP-Sepharose (0.5 × 2.6 cm) or 0.2 mL of dATP-Sepharose (0.4 × 1.6 cm) were first washed with four column volumes of 6 M guanidine hydrochloride (pH 7.0) containing 0.1% β-mercaptoethanol and then with 20 volumes of 50 mM Tris[1]-Cl buffer (pH 7.6)–10 mM $MgCl_2$–2 mM dithiothreitol. This buffer was used throughout all experiments with the additions indicated.

Protein B1, which had been stored at −70 °C in 20% glycerol–50 mM Tris-Cl buffer (pH 7.6)–10 mM $MgCl_2$–10 mM dithiothreitol (20 mg of protein per mL), was diluted with 50 mM Tris-Cl (pH 7.6)–10 mM $MgCl_2$–2 mM dithiothreitol to a final concentration of approximately 1 mg/mL and applied to the columns. These were then washed with three column volumes of the same buffer followed by five volumes of the same buffer containing 0.2 M NaCl. This procedure removed all unspecifically bound protein. Finally, three column volumes of the buffer without NaCl were passed through the columns.

Elution of protein B1 was performed with linear concentration gradients of nucleoside triphosphates in 50 mM Tris-Cl buffer (pH 7.6)–10 mM $MgCl_2$–2 mM dithiothreitol. In all experiments with the dTTP-Sepharose column the gradients had total volumes of 10 mL and the rate of elution was 2.5 mL/h. For the dATP column the total volumes of the gradients were 4 mL and the elution rate was 0.8 mL/h. Between 20 and 30 fractions were collected.

The concentration of the eluting nucleotide was determined in each fraction using ^3H- or ^{32}P-labeled nucleotides and counting 20- or 50-μL aliquots in 100 μL of water plus 3 mL of Instagel (Packard). The eluting concentration of the nucleotide is defined as the concentration of the nucleotide in the peak fraction of protein B1 activity.

Assay for Protein B1. Protein B1 was localized by assaying its activity with the NADPH oxidation assay (Brown et al., 1969a) under the same conditions as before (von Döbeln and Reichard, 1976). Substrate and allosteric effector were chosen to obtain maximal activity depending on which nucleotide was used in the eluting gradient (Larsson and Reichard, 1966a,b). The substrate concentrations were always saturating (0.56 mM). One unit of protein B1 is that amount which catalyzes the reduction of 1 nmol of CDP/min with ATP as effector.

Results

Chromatography of Protein B1 on dTTP-Sepharose. First the maximal amount of protein B1 that could be bound to the dTTP column (0.5 mL) was tested. An excess of protein B1, 1800 units, was applied to the column, which was washed as described under Experimental Procedure. Unbound protein, 1400 units, was removed before the fourth column volume of buffer containing 0.2 M NaCl. Elution with 1 mM dTTP then released 460 enzyme units from the dTTP-Sepharose. Based on this determination, between 200 and 400 units of protein B1 were used for the following experiments. In all these, 70–85% of the applied B1 activity bound firmly to the dTTP-Sepharose column.

In the next experiment the eluting concentration of dTTP was determined by chromatography of protein B1 with a gradient from 0 to 20 μM [^3H]dTTP (Figure 1). The protein peak was recovered at 5.5 μM dTTP. Another experiment with a more shallow gradient (0 to 10 μM) gave an eluting concentration of 4.7 μM.

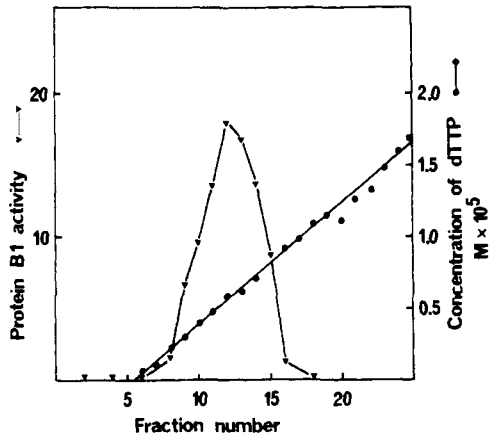

FIGURE 1: Elution of protein B1 from dTTP-Sepharose with dTTP. Protein B1 (250 units) was chromatographed on dTTP-Sepharose as described under Experimental Procedure. Elution was carried out with a gradient of 0 to 20 μM [^3H]dTTP (sp act. 8.4×10^4 cpm/μmol). Enzyme activity was measure as described in the Experimental Procedure with 0.10 mM dTTP as effector and CDP as substrate. Protein B1 activity is given as nanomoles of NADPH oxidized per minute and fraction under these conditions. The concentration of dTTP in the peak fraction of B1 activity was 5.5 μM.

Similar experiments were carried out with linear gradients of dATP, dGTP, ATP, and dCTP. All these nucleotides eluted B1 from the dTTP column, demonstrating that they competed with dTTP for binding sites on the protein. Of the five nucleotides tested, dATP eluted protein B1 at the lowest concentration (1.8 μM), while higher concentrations were needed for dGTP, dTTP, and dCTP (2.9, 5.1, and 47 μM, respectively). The concentration of ATP (122 μM) needed to elute the protein from the column was approximately 100-fold higher than that of dATP. It is therefore concluded that dATP has the highest and ATP the lowest affinity for the sites on protein B1 via which it is bound to the dTTP-Sepharose.

Correlation between Eluting Concentrations and K_{diss} Values for the Effectors. An empirical linear correlation between eluting concentrations of coenzymes and dissociation constants for coenzyme–enzyme binary complexes has been described for some dehydrogenases, which were chromatographed on an immobilized AMP analogue (Brodelius and Mosbach, 1976).

By analogy, the above chromatography of ribonucleotide reductase on immobilized dTTP makes it possible to determine unknown dissociation constants for nucleoside triphosphate effectors of this enzyme. As shown in Figure 2, a straight line was drawn in a plot of the eluting concentrations determined with the dTTP column as a function of the corresponding dissociation constants for the effectors obtained earlier from binding studies using dialysis techniques by Brown and Reichard (1969). Using the equation for the line ($Y = 12X + 1.7 \times 10^{-6}$), the dissociation constant for ATP was calculated to be 10 μM, which is in good agreement with the approximate value of 10 μM obtained by Brown and Reichard (1969). The previously unknown dissociation constant for dCTP was calculated in the same way to be 4 μM.

Chromatography on dATP-Sepharose. As mentioned earlier, the presence of high concentrations of dATP inhibits ribonucleotide reductase. Under these conditions the active 1:1 complex between proteins B1 and B2 aggregates to form inactive dimers and higher order complexes (Thelander, 1973).

[1] Abbreviation used is: Tris, tris(hydroxymethyl)aminomethane.

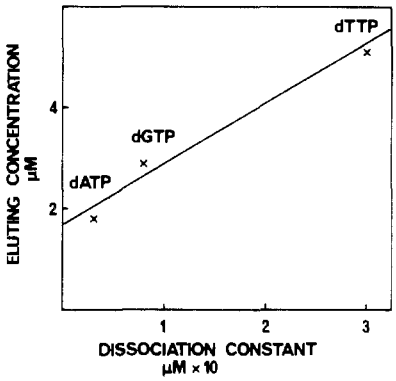

FIGURE 2: Eluting nucleoside triphosphate concentrations as a function of dissociation constants for the binding of nucleoside triphosphate effectors to protein B1. Experiments were performed with the dTTP-Sepharose column as described under Experimental Procedure. Measurements of protein B1 activity in the eluted fractions were carried out with the following combinations of allosteric effector and substrate: for dGTP elution, 0.1 mM dGTP and 0.56 mM GDP; for ATP, dATP, and dCTP elution, 1.5 mM ATP and 0.56 mM CDP. (No influence on the rate of CDP reduction by the concentrations of dATP or dCTP obtained in the testing cuvettes was observed in controls with and without these effectors present at this concentration of ATP; not shown.) For dTTP elution the assay conditions described in Figure 1 were used. The dissociation constants are taken from earlier data (Brown and Reichard, 1969).

Thus, the inhibition of ribonucleotide reductase seems to occur by a mechanism different from the stimulation by positive effectors. The dATP binding sites of protein B1 were studied by chromatography on dATP-Sepharose.

The B1 binding capacity of the dATP column (0.2 mL) was determined in the same way as described for the dTTP column. The dATP column could bind 530 units of B1 and therefore 300–450 units were used in the subsequent experiments. Under these conditions all of the applied B1 activity bound firmly to the dATP-Sepharose.

The eluting concentration of dATP in chromatography of B1 on the column was 20 μM, as determined with a gradient from 0 to 60 μM dATP (Figure 3a). This is, however, an approximate value, since binding of dATP from the buffer to protein B1 distorts the gradient. Also the pyrimidine specific positive effector of ribonucleotide reduction, ATP, eluted B1 from dATP-Sepharose and the eluting concentration of this nucleotide was 0.18 mM (Figure 3b).

In contrast neither dTTP nor dGTP could release the protein from the dATP-Sepharose. Using 5 mM dTTP only 9% of the applied B1 activity was eluted. The corresponding figure obtained with 5 mM dGTP was 18%. These data indicate that dTTP and dGTP do not bind to the same sites on protein B1 as dATP. The finding that dATP eluted B1 from the dTTP column demonstrated that dATP binds to the sites which bind dTTP. The inability of dTTP or dGTP to elute the protein from dATP-Sepharose can be explained by the presence of more binding sites for datp than for dTTP on the B1 molecule.

To test this hypothesis, elution of B1 from dATP-Sepharose was performed with ATP gradients containing a constant high concentration of dTTP or dGTP. Figures 3c and d show elution patterns with gradients of 0–0.5 mM ATP with 0.5 mM dTTP or 0.5 mM dGTP present throughout the gradients in the re-

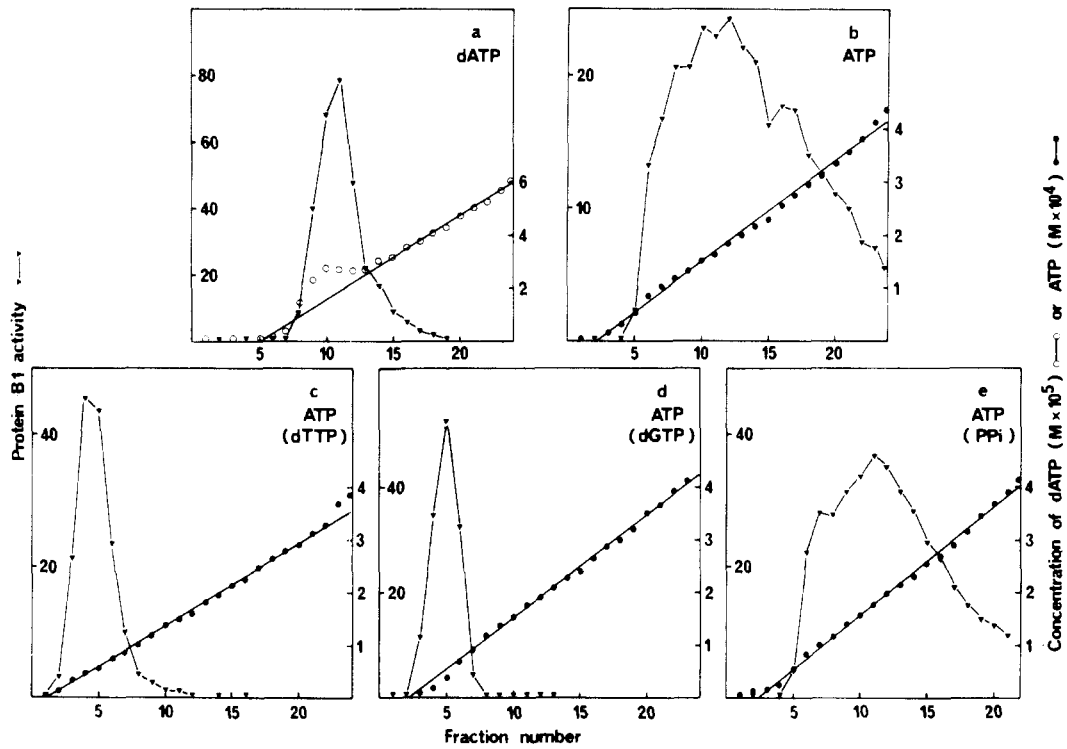

FIGURE 3: Chromatography of protein B1 on dATP-Sepharose. Experiments were performed as outlined in the Experimental Procedure. Protein B1 was assayed with 1.5 mM ATP and 0.56 mM CDP except as indicated in c and d, and protein B1 activity is given as nanomoles of NADPH oxidized per minute and fraction. (a) Elution with a gradient of 0–60 μM [^{3}H]dATP (sp act. = 6.3 × 10^5 cpm/μmol). (b) Elution of protein B1 with a gradient of 0–0.5 mM [α-^{32}P]ATP (sp act. 2.2 × 10^5 cpm/μmol). (c) Protein B1 elution with 0–0.5 mM [α-^{32}P]ATP with 0.5 mM dTTP present throughout the gradient. The experiment was performed as described under Experimental Procedure but before elution was started the column was washed with 5 × 0.2 mL of 5 mM dTTP followed by 4 × 0.2 mL of 0.5 mM dTTP. The wash fractions containing dTTP were assayed for protein B1 activity with CDP and 0.1 mM dTTP. The four first fractions from elution with 5 mM dTTP contained altogether 9% of the B1 activity applied to the column. The fractions from the gradient elution were assayed with CDP and 7.7 mM ATP. (d) Elution with ATP gradient containing 0.5 mM dGTP. The experiment was carried out in the same way as in c, except for the substitution of dGTP for dTTP throughout the gradient. The fractions from elution with dGTP alone were assayed with 0.1 mM dGTP as effector and GDP as substrate and contained 18% of the B1 activity applied to the column. (e) Elution with ATP gradient containing 1 mM pyrophosphate.

spective cases. As seen in these figures, the eluting concentration of ATP was lower in the presence than in the absence of either of the other effectors (cf. Figure 3b). This result could not be explained simply by the increase in ionic strength, since addition of 1 mM pyrophosphate to the ATP gradient did not influence the eluting concentration of ATP (0.18 mM) (Figure 3e).

Discussion

Based on dialysis binding experiments with radioactively labeled nucleoside triphosphates, Brown and Reichard earlier proposed a model for the binding of allosteric effectors to ribonucleotide reductase (Brown and Reichard, 1969). Their results indicated that the allosteric effectors bind exclusively to protein B1 and to four sites which are discrete from the substrate binding sites. According to the model, the effector sites consist of two classes of two sites each. One class was called h-sites because it bound dATP with a high affinity and the second class l-sites because it bound dATP with a lower affinity. Competition experiments indicated that the h-sites could bind all effectors (dATP, dGTP, dTTP, and ATP) and they were therefore thought to regulate the substrate specificity of ribonucleotide reductase. The l-sites could only bind dATP or ATP and were therefore assumed to regulate the overall activity of the enzyme.

Chromatography of protein B1 on immobilized effectors was carried out to test this model with an independent technique.

Experiments with dTTP-Sepharose demonstrated that the binding sites for dTTP on protein B1 can bind all the other allosteric effectors (dATP, dGTP, ATP, or dCTP), since these nucleotides eluted B1 from the dTTP column. In the experiments with this column between 15 and 30% of the applied protein B1 activity was eluted during the washing procedure, while all B1 activity bound firmly to the dATP column. The allosteric regulation of CDP reduction catalyzed by this unbound fraction was not impaired (data not shown). The reason for the inability of it to bind to the dTTP-Sepharose has not been investigated further.

The eluting concentrations obtained for the different allosteric effectors could be used to calculate unknown dissociation constants for effector binding to B1. A plot of the eluting concentrations as a function of dissociation constants determined earlier was linear (Figure 2). The line does not pass through the origin, however. A possible explanation for this intercept might be that the protein, in addition to specific binding to the immobilized dTTP, also binds unspecifically to the Sepharose matrix or to the *p*-aminophenyl group, perhaps by hydrophobic forces. Using the equation of the line the unknown dissociation constant for dCTP was calculated from its eluting concentration. The value obtained (4 μM) indicates that dCTP binds to protein B1 with a lower affinity than dTTP or dGTP. This is in accordance with kinetic experiments, where dCTP stimulates the reduction of GDP. However, higher concentrations of dCTP than of dTTP or dGTP are needed for the same stimulation (Larsson and Reichard, 1966b).

Chromatography of protein B1 on dATP-Sepharose gave more complex results. First of all, ATP binds to the dATP binding sites on protein B1. This statement is based on the fact that ATP eluted the protein from the dATP column. Secondly, dTTP or dGTP did not elute protein B1 from dATP-Sepharose, indicating that these nucleotides do not bind to the dATP–ATP binding sites. On the other hand, dATP eluted B1 from the dTTP column. These experiments together suggest that dATP binds to the sites binding dTTP (which bind all effectors) and in addition to more sites, which have the capacity to also bind ATP. This assumption is further supported by the elution experiments of B1 from the dATP column with ATP gradients containing dTTP or dGTP. When high concentrations of dTTP or dGTP were present to saturate the sites which can bind all effectors, protein B1 was eluted at lower ATP concentrations than with ATP alone (Figure 3). Thus, both dTTP and dGTP could compete out dATP from the sites binding all effectors, leaving the enzyme bound only via the dATP–ATP binding sites. Moreover, the elution of protein B1 at lower ATP concentrations when dTTP or dGTP was present than in the absence of these effectors indicates that the dATP–ATP binding sites have lower affinity for dATP, at least when the sites binding all effectors are occupied.

The experiments reported here provide further support to the model presented earlier, according to which protein B1 has two classes of effector binding sites: one class which binds all effectors (h-sites binding dATP, dTTP, dGTP, or ATP) and a second class binding only dATP or ATP (l-sites).

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