Inhibition of Azotobacter vinelandii RNA Polymerase by Cibacron Blue F3GA*

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Cibacron blue F3GA is a potent inhibitor of the Azotobacter vinelandii DNA-directed RNA polymerase. Addition of 8 \( \mu \text{M} \) Cibacron blue F3GA prior to initiation results in a greater than 90% inhibition of the poly[d(A-T)]-directed synthesis of poly[r(A-U)] while addition of the dye during the course of the reaction is without effect on chain elongation. Binding of RNA polymerase to [\( ^{3} \text{H} \)]poly[d(A-T)] is inhibited by only 15% in the presence of 8 \( \mu \text{M} \) Cibacron blue F3GA. Inhibition by Cibacron blue F3GA is noncompetitive with regard to ATP, UTP, or template. The poly[d(A-T)]-directed pyrophosphate exchange reaction is relatively resistant to inhibition by Cibacron blue F3GA. Rifampicin added to a similar reaction (in the presence or absence of Cibacron blue F3GA) results in a 95% inhibition of the exchange reaction. The interaction of the RNA polymerase core enzyme with Cibacron blue F3GA is shown by the formation of a difference spectrum with a positive maximum at 675 nm which is not affected by the presence of a high concentration (4 \( \mu \text{M} \)) of rifampicin. The data indicate that Cibacron blue F3GA acts by binding to RNA polymerase and inhibits a step between the synthesis of the initial phosphodiester bond and formation of a stable ternary elongation complex.

Cibacron blue F3GA, a sulfonated polyaromatic dye, has been shown to bind to several enzymes which interact with nucleotide substrates or nucleotide coenzyme ligands (1-3). Thompson et al. (1) and Thompson and Stellwagen (2) have proposed that the dye binds to the dinucleotide fold present in these proteins. DNA-dependent RNA polymerase can be envisaged to contain one or more such sites because of the number of nucleotide binding sites present on the enzyme such as the template binding region (4), product binding site (5), and initiation and elongation nucleotide binding sites (6-8). The present report describes the effects of Cibacron blue F3GA on RNA polymerase from Azotobacter vinelandii. The results to be presented indicate that Cibacron blue inhibits RNA polymerase at a step subsequent to the synthesis of the initial phosphodiester bond and before the formation of a stable elongation complex.

MATERIALS AND METHODS

Unless otherwise stated, all biochemicals used were of highest purity obtained either from Sigma or P-L Biochemicals. [\( ^{3} \text{H} \)]Poly[d(A-T)] and [\( ^{3} \text{H} \)]poly[d(U-U)] were prepared using DNA polymerase I (9). Cibacron blue F3GA was a generous gift from Dr. H. Bosshard of CIBA-GEIGY Ltd., Basel, Switzerland. Aqueous solutions of required concentrations of the dye were prepared using an extinction coefficient of 13.6 \( \text{mM}^{-1} \text{cm}^{-1} \) at 610 nm (2). DNA-dependent RNA polymerase from Azotobacter vinelandii was prepared by a modification of the published procedure (10). Holoenzyme and core RNA polymerases were resolved by gradient elution from polyacrylamide gel electrophoresis. Nitrocellulose membrane filters (0.45 \( \mu \text{m} \), 25-mm diameter) were obtained from Matheson-Higgins Co. Prior to use, the filters were soaked in 0.1 \( \text{M} \) KOH for 30 min at room temperature.

Enzyme Assays: Template-dependent Polymerization—Synthesis of polypeptideoligonucleotides was followed by measuring the amount of an appropriate [\( ^{3} \text{H} \)]ribonucleotide (2000 to 4000 cpm/nmol) incorporated into trichloroacetic acid-precipitable material. The composition of reaction mixtures and experimental conditions are described in the text. Reactions were stopped by the addition of 3 ml of cold 5% trichloroacetic acid. The mixture was filtered onto Whatman GF/C discs (presoaked in 0.01 \( \text{M} \) ATP), washed with 3 \( \times \) 5 ml of 5% trichloroacetic acid and dried, and placed in vials containing 5 ml of 4.2% liquid scintillator. Radioactivity was measured in a Beckman LS-280 liquid scintillation counter.

Template-dependent Pyrophosphate Exchange—The [\( ^{32} \text{P} \)]pyrophosphate (3000 to 4000 cpm/nmol) exchange reaction was carried out essentially according to the procedure of Krakow and Pronk (11). The composition of reaction mixtures is described in the text. Reactions were terminated by the addition of 0.1 ml of 0.5 \( \text{M} \) EDTA, 9.1 ml of 0.2 \( \text{M} \) NaPP, (pH 6), and 0.5 ml of a 10% suspension of activated charcoal (Norit) in 0.01 \( \text{M} \) NaPP, (pH 6). The mixture was filtered onto Whatman GF/C discs (presoaked in 0.01 \( \text{M} \) sodium pyrophosphate) washed three times with 5 ml portions of 0.01 \( \text{M} \) NaPP, (pH 6) and dried and the radioactivity was measured in a Nuclear Chicago gas-flow counter.

Template Binding Assay—The binding of RNA polymerase to [\( ^{3} \text{H} \)]poly[d(A-T)] was assayed by measuring the radioactivity retained on nitrocellulose membranes (12).

RESULTS

The results of an experiment in which [\( ^{3} \text{H} \)]poly[d(A-T)] binding and the synthesis of poly[r(A-U)] directed by poly[d(A-T)] are measured as a function of the concentration of Cibacron blue are presented in Fig. 1. It is evident that the synthesis of poly[r(A-U)] is much more sensitive to inhibition by Cibacron blue than is the binding of [\( ^{3} \text{H} \)]poly[d(A-T)] by RNA polymerase. Polynucleotide synthesis was almost completely inhibited by 8 \( \mu \text{M} \) Cibacron blue while the binding of poly[d(A-T)] was inhibited by only 15% at this dye concentration. As the dye concentration was increased, an increased inhibition of poly[d(A-T)] binding occurred and at 32 \( \mu \text{M} \) Ciba-
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Cibacron blue only 40% of the polymerase-poly(dA-T) complex remained.

To determine whether the inhibition of RNA polymerase by Cibacron blue was template- or product-dependent, a variety of DNA template-directed reactions were assayed. The data presented in Table I show that while the synthesis of complementary polynucleotides was inhibited by Cibacron blue the relative sensitivity varied with the template used and cannot be readily explained by the product sequence. While the poly(dC)-directed synthesis of poly(rG) is 95% inhibited by 4 μM Cibacron blue, the synthesis of poly(rA) directed by poly(dA-dT) is relatively resistant to this concentration of the dye. In contrast, the synthesis of poly(rA) directed by either single-stranded poly(dT) or double-stranded poly(dA-dT) is almost equally inhibited by 4 μM Cibacron blue. At a concentration of 8 μM Cibacron blue all the polymerization reactions studied were inhibited by 90% or more.

The inhibition by Cibacron blue of the poly(dA-T)-directed synthesis of poly(rA-U) was not reversed by increasing concentrations of poly(dA-T). A double reciprocal plot of the rate of poly(rA-U) synthesis versus poly(dA-T) concentration shows a pattern typical of noncompetitive inhibition (Fig. 2A). Using poly(dA-T) as template, the effect of nucleotide concentration on Cibacron blue inhibition was determined. At a saturating concentration of either ATP or UTP (0.4 mM), variation of the concentration of the alternate ribonucleoside triphosphate in the presence and absence of Cibacron blue gave linear double reciprocal plots (Fig. 2B and C). Cibacron blue also appears to be a noncompetitive inhibitor with respect to the binding of ATP and UTP to RNA polymerase; the

**Table I**

Inhibition of template-directed polynucleotide synthesis by Cibacron blue F3GA

| Templates          | NTP        | [3H]NMP incorporated |
|--------------------|------------|----------------------|
|                    | None       | +4 μM CB             | +8 μM CB          |
| Poly(dA-T)         | [3H]UTP + ATP | 5.41                | 1.32               | 0.45                |
| Poly(dT)           | [3H]ATP    | 3.24                 | 0.14               | 0.01                |
| Poly(dA-dT)        | [3H]ATP    | 2.31                 | 0.13               | 0.09                |
| Poly(dC)           | [3H]GTP    | 6.56                 | 0.32               | 0.16                |
| Poly(dI-dC)        | [3H]GTP    | 6.37                 | 0.24               | 0.14                |
| Poly(dI-C)         | [3H]GTP    | 6.39                 | 0.29               | 0.23                |
| Calf thymus DNA    | [3H]UTP + ATP | 2.78                | 0.89               | 0.23                |
| DNA                | CTP, GTP   |                      |                     |                     |

The reactions contained (final volume 0.25 ml) and the components were added in the following sequence: 80 mM Tris/HCl, pH 7.8, 40 mM mercaptoethylamine, 20 mM MgCl₂, 3 μg of RNA polymerase core enzyme, the indicated concentrations of Cibacron blue (CB), DNA (9 nmol of the indicated polydeoxynucleotide or 40 μg of calf thymus DNA), 0.4 mM NTP as indicated. (The specific activity of the [3H]NTP used was from 2000 to 4000 cpm/nmol.) The incubation was for 10 min at 37°C.

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**Fig. 1.** Effect of Cibacron blue F3GA on [3H]poly(dA-T) binding to RNA polymerase and on poly(dA-T)-directed synthesis of poly(rA-U). Binding: additions to reaction mixture (final volume 0.25 ml) were in the following order: 80 mM Tris/HCl, pH 7.8, 3 μg of RNA polymerase core enzyme, indicated concentrations of Cibacron blue, and 9 nmol of [3H]poly(dA-T) (2262 cpm/nmol). Incubations were for 5 min at 37°C. Each reaction mixture was filtered through a nitrocellulose disc and washed with 2 ml of a solution containing 0.02 M Tris/HCl, pH 7.0, 0.05 M sodium chloride, and Cibacron blue of the same concentration as the incubation. The discs were dried and radioactivity was measured. (2782 cpm were retained on the nitrocellulose filter in the control which did not contain Cibacron blue.) Polymerization: the composition of reaction mixture and order of additions were as given for the poly(dA-T) template in Table I. Incubations were for 10 min at 37°C. (5.4 nmol of [3H]UMP were incorporated into poly(rA-U) in the control reaction.) ○—○, poly(dA-T) binding; △—△, incorporation of [3H]UMP into poly(rA-U).

**Fig. 2.** Double reciprocal plots of substrates and template versus velocity in a poly(dA-T)-directed reaction in the presence and absence of Cibacron blue F3GA. The reaction mixtures and conditions of assay were as given in Table I except that the reaction mixtures (total volume, 0.25 ml) contained 80 mM Tris/HCl, pH 7.8, 40 mM mercaptoethylamine, 20 mM MgCl₂, 3 μg of RNA polymerase core enzyme, 2 or 4 μM Cibacron blue F3GA, 9 nmol of poly(dA-T), 0.4 mM UTP, and 0.4 mM ATP. Incubations were for 10 min at 37°C. A, and C, [3H]UMP (2000 cpm/nmol) incorporation was measured and the concentrations of ATP and poly(dA-T), respectively, were varied; and for B, [3H]AMP (3145 cpm/nmol) incorporation was measured and the concentration of UTP was varied. ○—○, no Cibacron blue; △—△, 2 μM Cibacron blue; △—△, 4 μM Cibacron blue.
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Apparent $K_m$ remained unchanged while the $V_{max}$ decreased in the presence of the inhibitor. Assuming a noncompetitive inhibition, the apparent $K_v$ value for Cibacron blue was 2 $\mu$M.

The sensitive step for inhibition by Cibacron blue lies between template binding and chain elongation. Addition of 8 $\mu$M Cibacron blue prior to incubation (Fig. 3) resulted in an almost complete inhibition of the poly[d(A-T)]-directed synthesis of poly[r(A-U)]. Addition of 8 $\mu$M Cibacron blue at 5 min after the beginning of the reaction showed that the dye did not inhibit RNA polymerase in the ternary elongation complex. The divergence between the control and the reaction to which Cibacron blue was added at 5 min may be a consequence of inhibition of reinitiation by RNA polymerase. The reactions directed by the single-stranded templates, poly(dT) and poly(dC), are also refractory to inhibition by Cibacron blue added during chain elongation (data not shown).

The poly[d(A-T)]-directed exchange of [38P]PPi into UTP in the presence of catalytic levels of ATP (4 $\mu$M) provides a convenient assay for events occurring during and immediately after chain initiation. The reaction initially takes place at the level of the initiating dinucleoside tetraphosphate and continues during chain elongation.

$$38P + ATP = 38PAP + PP_i$$

The effect of 8 $\mu$M Cibacron blue on the poly[d(A-T)]-directed pyrophosphate exchange reaction is shown in Fig. 4. RNA polymerase holoenzyme was preincubated with the dye prior to addition of poly[d(A-T)] and the substrates; under these conditions the synthesis of poly[r(A-U)] is only about 5% of the control (Fig. 3). In distinct contrast to polymerization, the pyrophosphate exchange reaction is relatively insensitive to Cibacron blue. The reaction containing 8 $\mu$M Cibacron blue and 4 $\mu$M ATP + 0.4 mM UTP showed about 80% of the exchange activity of the control, while the reaction containing 8 $\mu$M Cibacron blue and 0.4 mM ATP + 0.4 mM UTP retained about 65% of the control activity. The insensitivity of RNA polymerase engaged in the pyrophosphate exchange reaction to inhibition by Cibacron blue was not due to the presence of pyrophosphate. The concentration of pyrophosphate used in the exchange reaction inhibits polymerization by 50% (Table II). In the presence of 1 mM sodium pyrophosphate, addition of Cibacron blue still resulted in the inhibition of poly[r(A-U)] synthesis.

The relatively lower sensitivity of the pyrophosphate exchange reaction to inhibition is also shown by replacing ATP with 5'-AMP or UpA; under these conditions only a single phosphodiester bond can be synthesized with UTP present as the only ribonucleoside triphosphate. As shown in Table III, the single step addition and pyrophosphorolysis reaction with UTP and 5'-AMP or UpA was also relatively insensitive to Cibacron blue. The poly[d(I-C)]-directed pyrophosphate exchange experiment was carried out in the presence of 4 $\mu$M ATP and 0.4 mM UTP or 0.4 mM ATP and 1 mM [38P]ppi.

The following reaction mixture (additions shown in parentheses) was incubated at 37°C for 40 min after chain initiation. The first addition of the dye was made at 5 min. The inhibition of reinitiation by RNA polymerase is also shown by replacing ATP with 5'-AMP or UpA, under these conditions only a single phosphodiester bond can be synthesized with UTP present as the only ribonucleoside triphosphate. As shown in Table III, the single step addition and pyrophosphorolysis reaction with UTP and 5'-AMP or UpA was also relatively insensitive to Cibacron blue.

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TABLE III

| Template Additions | [32P]PP′ incorporated |
|-------------------|----------------------|
|                   | None 8 μM CB 16 μM CB |
| A. Poly(d(A-T))   | ATP (4.4 mM) + UTP   |
|                   | 10.0 7.8 4.3         |
|                   | ATP (0.4 mM) + UTP   |
|                   | 2.2 2.2 1.2          |
|                   | AMP (0.4 mM) + UTP   |
|                   | 2.3 1.4 0.95         |
|                   | UpA (0.4 mM) + UTP   |
|                   | 5.2 2.7 1.16         |
|                   | ATP (0.4 mM) + UTP′  |
|                   | 0.6 0.43             |
|                   | rifampicin (4 μM)    |
|                   | 500 250 100          |
| B. Poly(d(I-C))   | GTP (3.2 μM) + CTP   |
|                   | 9.8 5.2 2.9          |
|                   | GTP (0.4 mM) + CTP   |
|                   | 3.2 1.8 0.4          |

change reaction was also less inhibited by Cibacron blue than was the synthesis of poly[r(G-C)]. In the presence of 16 μM Cibacron blue, the synthesis of poly[r(A-U)] or poly[r(G-C)] would be almost completely inhibited. As shown in Table III, even at the high inhibitor concentration considerable pyrophosphate exchange activity remained. Both Cibacron blue and rifampicin (13) inhibit RNA polymerase when added prior to chain initiation and the ternary elongation complex is not affected by either inhibitor. As indicated in Table III, the pyrophosphate exchange reaction is relatively insensitive to Cibacron blue but is markedly, but not completely, inhibited by rifampicin.

The data indicate that Cibacron blue is a noncompetitive inhibitor which acts by binding to a site (or sites) on the RNA polymerase core unit. Thompson and Stellwagen (2) have shown that the absorption spectrum of Cibacron blue bound to protein undergoes a red shift. As shown in Fig. 5, addition of Cibacron blue to RNA polymerase produced difference spectra with positive absorption maxima at 675 nm and an isosbestic point at 585 nm. The increase in the absorbance at 675 nm exhibits a hyperbolic dependence on the concentration of the added dye (Fig. 5) indicating the formation of a saturated Cibacron blue–polymerase complex.

Using a saturating concentration of rifampicin (20 μM) to form the rifampicin–polymerase complex, the subsequent addition of 8 μM Cibacron blue still produced the characteristic difference spectrum with a maximum at 675 nm (results not shown). This and the data presented in Table III indicate that each ligand binds to a separate site on the core enzyme.

**DISCUSSION**

The nature of the inhibition of RNA polymerase produced by Cibacron blue is in several respects similar to that of rifampicin. Inhibition is noncompetitive with respect to template and substrates (Fig. 2, A, B, and C) and RNA polymerase engaged in the ternary elongation complex is not inhibited by Cibacron blue (Fig. 3) or by rifampicin (13). In each case, synthesis of at least the first phosphodiester bond is not blocked by the inhibitor. Johnston and McClure (14) have shown that the initial dinucleoside tetraphosphate is still synthesized by promoter–poly(d(A-T))-bound RNA polymerase in the presence of rifampicin. While we have not as yet determined the nature of the product synthesized in the presence of high concentrations of Cibacron blue, the ability of the "inhibited" enzyme to catalyze the pyrophosphate exchange reaction is evidence for the formation of at least the first phosphodiester bond. That the mechanisms by which rifampicin and Cibacron blue inhibit RNA polymerase differ in detail is indicated by the marked inhibition of pyrophosphate exchange by rifampicin. We have carried out Cibacron blue challenge experiments analogous to the rifampicin challenge procedure of Mangel and Chamberlin (15) using the poly(d(A-T))-directed reaction and found that inhibition was instantaneous, suggesting that a different substep in the initiation reaction leading to the resistant elongation complex was affected by Cibacron blue. The noncompetitive nature of the inhibition by the dye and the demonstration that the synthesis and pyrophosphorylation of at least the initial dinucleoside tetraphosphate can occur in the presence of Cibacron blue suggest that the sensitive step may be translocation. This is also the mechanism suggested for inhibition of RNA polymerase by rifampicin (14) and it is possible that Cibacron blue may prevent continued chain elongation by occluding the RNA product site. We are currently characterizing the products synthesized in the presence of Cibacron blue to determine whether a discrete size class is formed i.e. dinucleoside tetraphosphate or longer.

Thompson and Stellwagen (2) have proposed that the forma-
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The inhibition of RNA polymerase by Cibacron Blue is characterized by a difference spectrum having a positive maximum in the range 660 to 680 nm. This suggests the complexation of Cibacron Blue with proteins containing a secondary structure termed the dinucleotide fold (16, 17). The RNA polymerase core enzyme-dye complex produces a difference spectrum having a positive maximum at 675 nm. This indicates the presence of a dinucleotide fold in RNA polymerase and at least a hydrophobic pocket which the dye occupies to produce a perturbation in the absorption spectrum of the dye chromophore.

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Several dyes have now been shown to inhibit RNA polymerase by directly complexing with the enzyme. Congo red (19) and gallin (20) block template binding. Cibacron blue inhibits at a step subsequent to synthesis of the initial dinucleoside tetraphosphosphate and before formation of a ternary elongation complex. Rose bengal has been shown to be an inhibitor of chain elongation (21). It appears probable that other dyes will be found to inhibit this enzyme and may allow for a further dissection of the substeps involved in the RNA polymerase reaction.

Since the completion of this study we have found that the wheat germ RNA polymerase II (22) is inhibited by Cibacron Blue F3GA and binds to Cibacron blue/Sepharose.2

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Several dyes have now been shown to inhibit RNA polymerase by directly complexing with the enzyme. Congo red (19) and gallin (20) block template binding. Cibacron blue inhibits at a step subsequent to synthesis of the initial dinucleoside tetraphosphosphate and before formation of a ternary elongation complex. Rose bengal has been shown to be an inhibitor of chain elongation (21). It appears probable that other dyes will be found to inhibit this enzyme and may allow for a further dissection of the substeps involved in the RNA polymerase reaction.

Since the completion of this study we have found that the wheat germ RNA polymerase II (22) is inhibited by Cibacron Blue F3GA and binds to Cibacron blue/Sepharose.2

2 R. Doi, personal communication.
3 E. Eilen, S. Pawar, S. A. Kumar, and J. S. Krakow, unpublished results.
Inhibition of Azotobacter vinelandii RNA polymerase by cibacron blue F3GA.
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