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SUBUNIT CONTACTS OF THE RIFAMYCIN BINDING SITE OF RNA POLYMERASE (B. SUBTILIS)

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Summary: The radiolabeled RNA polymerase inhibitors 3-(2-bromoacetamidoethyl)-thiorifamycin and 3-(2-acetamidoethyl)-thiorifamycin quinone have been prepared and covalently attached to the B. subtilis enzyme under mild conditions. Analysis of the subunits labeled indicates that regions of subunits sigma, beta, and beta-prime lie within about 7Å of the 3-position of rifamycin bound to the enzyme, while subunits alpha, beta, and beta-prime lie near the aromatic rings. The results of this work imply that the rifamycin binding site lies near the center of an arrangement involving at least four of the subunits of RNA polymerase. This idea is supported by the results of other studies involving cross-linking of subunits and also rifamycin binding to subunit complexes.

Introduction: DNA-dependent RNA polymerase catalyzes the sequence of reactions known as transcription (1). As in other procaryotic RNA polymerases, RNA polymerase of B. subtilis is composed of five associated peptides; these are beta (M.W. = 140,000), beta-prime (130,000), sigma (55,000) and two alpha subunits (45,000).

The rifamycins are highly specific inhibitors of bacterial RNA polymerases, and are believed to prevent formation of the first or second phosphodiester bond in RNA synthesis (1,2). Studies with mutant enzymes suggest that the rifamycin binding site on E. coli RNA polymerase involves the beta subunit (3), and the results of Halling et al. (4,5) show similar properties for the B. subtilis enzyme.* An alkylating rifamycin derivative has been successfully used to probe the rifamycin binding site of E. coli RNA polymerase (6).

*In accordance with the recent work of Halling et al. (4,5) the zinc-containing subunit of B. subtilis is the second largest and is labeled beta-prime. The largest subunit is beta.

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Rifamycins, shown in Fig. 1, are strong inhibitors in either the quinone or hydroquinone form (7). Furthermore, it is possible that a quinone carbonyl can form a Schiff base with an amino group (8); it has been reported that rifampicin-quinone binds irreversibly to poly-L-lysine (9). Reduction of such a Schiff base with borohydride should lead to a stable amine product (10).

Successful chemical modification of a protein with a (haloacetyl) alkylating agent or with a reactive carbonyl compound depends on the presence of a nucleophilic group on the protein positioned appropriately for reaction. The two reagents discussed below possess different reactive groups in different locations, thus providing independent information about the subunits surrounding the rifamycin binding site. Comparison of results from enzymes of different species can provide additional knowledge, since the arrangement of nucleophiles on the enzymes may differ.

Materials and Methods: RNA polymerase from B. subtilis, prepared according to (5), was the kind gift of Dr. S. M. Halling and Prof. R. H. Doi. Protein concentrations were determined according to Bradford (11).

The buffers used were Buffer A: 40 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 8.0, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.5 M NaCl, 20% glycerol (v/v); and Buffer B: 40 mM phosphate buffer, pH 8.0, 0.1 mM EDTA, 0.5 M NaCl, 20% glycerol.

The synthesis of rifamycin derivatives requires rifamycin S (see Fig. 1B) as starting material. Oxidation of rifamycin SV into the S form by the method Sensi et al. (12) was used successfully only after the addition of vanadyl sulfate to the reaction mixture. Typically, 32 mg of vanadyl sulfate was added to a solution of 0.25 g rifamycin SV in 50 ml of anyhydrous methanol which was stirred vigorously at 4°C. This was followed by the addition of 2.5 ml of 30% H_2O_2 and left for 1/2 hr. In that time the dark, murky brown mixture became clear orange with a blue precipitate of excess vanadyl sulfate. The precipitate was filtered off and purification of product proceeded as described in (12). 3-(2-bromoacetamidoethyl)-thiorifamycin (BrRif) was prepared using a modification of the method of Stender, Stutz and Scheit (6). 3-(2-aminoethyl)-thiorifamycin was allowed to react with N-succinimidyl bromoacetate for one hour at room temperature, then excess solvent was evaporated off and the orange residue was taken up in 200 µl of benzene:acetone (2:1). The solution was then subjected to preparative thin-layer chromatography (TLC) on Merck silica gel plates in a benzene: acetone (1:1) solvent system. This resulted in four yellow spots (R_f = 0.07, 0.15, 0.43, 0.55). The hydroquinone and quinone products ($R_f \cong$ 0.15 and 0.55) were identified by a positive 4,4'-nitrobenzyl-pyridine test (14) and a negative ninhydrin test. In the case of the

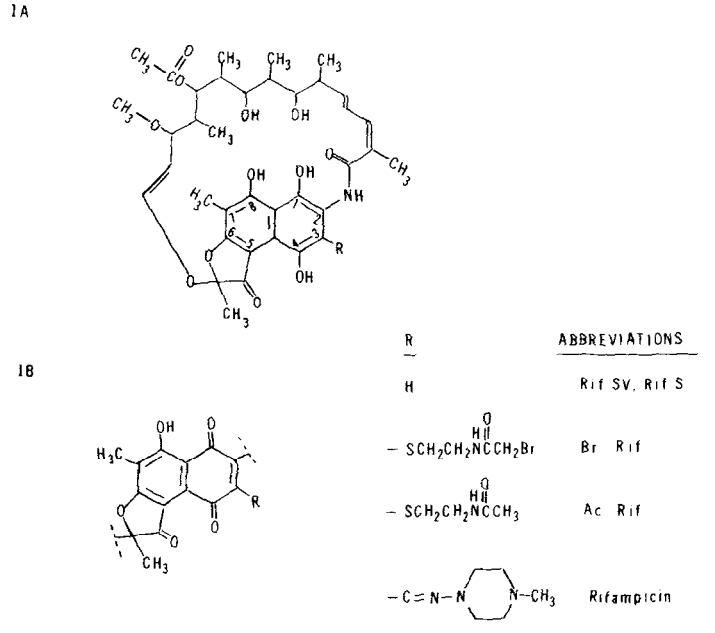

Figure 1. Structures of the rifamycins discussed in the text; (1A) hydroquinone form, (1B) quinone form.

labeled derivatives, sections of the TLC plate were scraped and counted. Products were also identified spectroscopically; the hydroquinone was found to correspond to R_f = 0.15 and the quinone to R_f = 0.55. The product spots were scraped off TLC plates and extracted with acetone. The acetone solution was evaporated to dryness and the residue was taken up in either dimethylformamide or in 65 mM phosphate buffer, pH 7.0. Concentrations were determined spectroscopically using literature values for the extinction coefficient (6) and a specific radioactivity of 12,882 cpm/nmole was found.

3-(2-acetamidoethyl)-thiorifamycin (AcRif) was prepared in essentially the same way. The reaction mixture was subjected to preparative TLC as described above, resulting in 3 major yellow spots (R_f = 0.10, 0.35, 0.55). The products (R_f = 0.10, 0.55) were identified spectroscopically, by a negative ninhydrin test, and by liquid scintillation counting. Concentrations were determined spectroscopically, and a specific radioactivity of 49,022 cpm/nmole was found.

Inhibition studies of RNA polymerase by BrRif, AcRif-quinone, or rifampicin were performed by preincubating each inhibitor with RNA polymerase (3.6 μM) in a 1:1 mole ratio for 10 minutes at room temperature. From the incubation mixture, 15 μl aliquots were withdrawn and assayed according to Fukuda and Doi (15).

In order to minimize the possibility of nonspecific binding, all reactions were carried out with the enzyme concentration in excess over the rifamycin concentration.

RNA polymerase and [^{14}C]BrRif in Buffer A were incubated at 37° for 5 hours. Reactions were terminated by the addition of mercaptoethanol.

The solutions were subjected to sodium dodecylsulfate-urea gel electrophoresis (16) with the modification of substituting N,N'-diallytartardiamide for N,N'-methylenebisacrylamide on a mole for mole basis in the running gel. The samples were applied to a 3 mm-thick slab gel and run at 60V for 5 hours. Gels were stained overnight as described in (16) and destained in an aqueous solution which was 7% acetic acid (v/v) and 25% methanol (v/v). This procedure washes out unbound rifamycin. Excellent separation of the subunits was afforded by use of these gels. There is a particularly striking separation between β and β', where the subunits were routinely 4 mm apart (R_f's: β = 0.41, β' = 0.52). The gels were cut in 1 mm slices and counted (17).

RNA polymerase and [^{3}H]AcRif-quinone were preincubated at 37°C for 10 min in Buffer B, then placed in an ice bath. The reaction mixture was then made 10^{-3}M in $NaBH_4$ which had been freshly prepared in cold Buffer B. After a five minute exposure at 0°, the solution was subjected to electrophoresis.

In order to learn whether the sigma subunit was still associated with the enzyme under the reaction conditions chosen, a small aliquot of enzyme in Buffer A was applied to a Bio-Gel P-300 column (0.7 cm x 6.4 cm). The column and the eluting buffer were kept at 37°C. The absorbance profile showed that almost all of the protein eluted in two void volumes. The first 12 fractions were concentrated then subjected to tube gel electrophoresis as described in (16). The stained gels were then scanned on a Gilford 250 spectrophotometer, and the peaks corresponding to sigma and alpha were cut out and weighed. The results showed that the sigma subunit co-chromatographed with the enzyme, implying that it was tightly bound.

Results: As shown in Table I, the rifamycin derivatives were found to be very effective inhibitors of B. subtilis RNA polymerase.

A typical labelling profile using [^{14}C] BrRif is shown in Fig. 2A. The beta and beta-prime subunits are approximately equally labelled. Table II shows the percent of the total bound label that each subunit carried. The overall yield from the reaction was found to be 18-20%.

Results of $NaBH_4$ reduction of the complex between the quinone form of [^{3}H] AcRif and RNA polymerase are given in Fig. 2B and Table II. Yields from this reaction were found to be 1-2%; a low yield is to be expected in view of the comparatively low reactivity of rifampicin-quinone with amino groups (9). There are three possible carbonyl groups of the compound which could form Schiff bases; the two quinone carbonyls and the carbonyl on the five-membered ring (see Fig. 1). Using the hydroquinone form of [^{3}H]AcRif, the enzyme-rifamycin complex was treated with $NaBH_4$. No labeling of any subunit was found (data not shown). This implies that the covalent attachment of AcRif-quinone to the enzyme

Table I. Inhibition Studies of RNA Polymerase[a]

Inhibitor	p moles Uridine Incorporated	% Inhibition
None	196	0
BrRif	7	96
AcRif-quinone	16	92
Rifampicin	59	70

a. Assays performed according to Fukuda and Doi (15), using equimolar amounts of enzyme and inhibitor at 0.4 μM final concentration.

involves a quinone carbonyl, and by inference a Schiff base intermediate.

In order to avoid nonspecific labelling, care was taken to insure that the enzyme concentration was always greater than that of the rifamycin. In addition, the RNA polymerase preparations contained a protein contaminant having a molecular weight of 90,000. As a control the gel fractions containing this protein were always counted; no labelling was observed.

Conclusions: The formation of a covalent bond between a protein subunit and a reactive rifamycin implies that the subunit is close to the rifamycin binding site. If no bond is formed, no conclusion can be drawn about the location of the subunit, since it may not have a nucleophile situated in a productive position.

Stender et al. (6) used the alkylating derivative BrRif to probe the subunit arrangement around the rifamycin binding site of E. coli RNA polymerase. They found that the beta and sigma subunits were modified by treatment with BrRif. In B. subtilis, it is mainly the beta and beta-prime subunits which become labelled under similar reaction conditions.

The use of $NaBH_4$ to covalently attach AcRif-quinone to RNA polymerase gave the striking result that the smallest subunit, alpha, was the most heavily labelled. The beta and beta-prime subunits also were labelled.

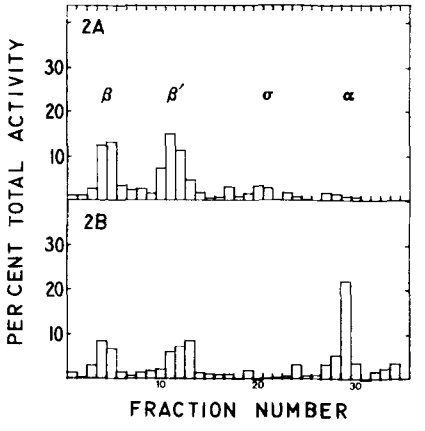

Figure 2. Percent Bound Radioactivity vs. Migration distance on SDS Polyacrylamide Gels. (A) Reaction of 0.70 μM BrRif with 0.81 μM RNA polymerase in Buffer A. (B) Reaction of 1.9 μM AcRif-quinone with 2.4 μM RNA polymerase and 1 mM $NaBH_4$ in Buffer B. Each fraction represents 1 mm; location of each subunit is indicated in figure.

Taken together, the results of the alkylation experiments suggest that the rifamycin substituent at position 3, which can sweep out a zone with a radius $\approx$ 7Å, contacts the sigma, beta, and beta-prime subunits. The experiments with the AcRif-quinone imply that the aromatic rings of rifamycin bind near the alpha, beta, and beta-prime subunits. These results are consistent with an arrangement of enzyme subunits such as that proposed recently by Hillel and Wu on the basis of crosslinking studies (18). They also illuminate the observation that rifamycin binds tightly to the $\alpha_2\beta$ complex (19,20).

The function of the alpha subunit is not yet known; however, chemical modification of alpha is known to alter the transcriptional specificity of RNA polymerase (21). The results of this work suggest that alpha may be located close enough to the active region of RNA polymerase to play a direct role in transcription. The evident central location of the rifamycin binding site with respect to the enzyme subunits, and the ease of preparation of new rifamycins, suggest that physical probe studies using rifamycin derivatives can provide further important information on the structure and function of RNA polymerase.

Table II. RNA Polymerase Subunits Modified[a]

Subunit	% Bound Inhibitor ± Standard Deviation	
	BrRif	AcRif-quinone
Beta	45 ± 11%	24 ± 2%
Beta-prime	35 ± 10	31 ± 4
Sigma	10 ± 2	6 ± 2
Alpha	10 ± 5	39 ± 7

a. Data for BrRif is from two experiments; data for AcRif-quinone is from 3 experiments.

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