Mechanism of action of nalidixic acid: Purification of *Escherichia coli* *nalA* gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme

(oxolinic acid/DNA relaxation/DNA supertwisting/ λ transducing phages/relaxation complex)

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ABSTRACT A target protein for nalidixic and oxolinic acids in *Escherichia coli*, the *nalA* gene product (Pnal), was purified to homogeneity as judged by gel electrophoresis, using an *in vitro* complementation assay. It is a dimer of identical 110,000-dalton subunits. A polypeptide of this molecular weight is uniquely induced by a λ *nalA* transducing phage, thereby showing that the purified Pnal is a product of the *nalA* gene. Nalidixic and oxolinic acids inhibit DNA gyrase activity and induce formation of a relaxation complex analogue. Treatment of the complex with sodium dodecyl sulfate causes a double-strand break in the DNA substrate and the resulting linear molecule seems covalently bound to protein. Complex formation, unlike the introduction of supertwists, does not require ATP or relaxed circular DNA and is insensitive to novobiocin. DNA gyrase from a strain with a *nalA* mutation conferring drug resistance (*nalA*^r) is 1/100 as sensitive to oxolinic and nalidixic acids with respect to inhibition of supertwisting and induction of the pre-linearization complex. Addition of Pnal restores drug sensitivity and stimulates DNA gyrase activity. DNA gyrase preparations and Pnal catalyze a third reaction sensitive to nalidixic and oxolinic acids, the ATP-independent relaxation of supertwisted DNA. Relaxation by gyrase from *nalA*^r cells is drug resistant. The nicking-closing activity is distinct from *E. coli* ω protein in several properties, including the ability to relax positively supertwisted DNA. We postulate that the *nalA* gene product occurs in two molecular forms, as Pnal and as a gyrase component. Both forms catalyze nicking-closing, and inhibition of this activity by nalidixic and oxolinic acids may account for the inhibition of DNA synthesis by these drugs.

Nalidixic acid (Nal) and oxolinic acid (Oxo) are widely used antibacterial agents which cause a preferential, rapid, and reversible inhibition of DNA synthesis (reviewed in ref. 1). The specificity is not absolute because RNA synthesis can be inhibited at high doses. The *nalA* gene probably codes for the target protein (Pnal), because mutations in this gene confer high level resistance in several *in vitro* DNA replication systems, including that of ϕ X174 replicative form I (RFI) replication (1, 2). To elucidate the mechanism of action of the drugs and the role of Pnal in DNA synthesis, we have extensively purified Pnal. While the study was in progress, M. Gellert communicated his preliminary experiments suggesting that Oxo inhibits DNA gyrase and can result in cleavage of the double-stranded DNA substrate. Gyrase introduces negative supercoiling into closed, circular DNA (3, 4), and is inhibited by another DNA synthesis inhibitor, novobiocin. Mutations conferring novobiocin resistance (*cou*^r), which are not in *nalA*, induce formation of a drug-resistant DNA gyrase (5). We have explored the relationship of Pnal and gyrase and the effects of the drugs on their activities. This report summarizes the identification, purification, and properties of Pnal; the inhibition of DNA gyrase

by Nal and Oxo; the drug-induced formation of a relaxation-type complex in the presence of DNA gyrase preparations; and the drug inhibition of a nicking-closing activity in Pnal and DNA gyrase preparations which is distinct from ω protein (6).

MATERIALS AND METHODS

Bacteria. The *Escherichia coli* strains, H560 (*polA*, *endA*) and H560-1 (*polA*, *endA*, *nalA*^r), were from R. Sternglanz.

Chemicals. Agarose (type II), spermidine-HCl, Nal, and novobiocin were from Sigma Chemical Co. Hydroxylapatite (Bio-Gel HTP) was from Bio-Rad Laboratories. DEAE-cellulose (DE52) and phosphocellulose (P11) were purchased from Whatman. Sephadex G-200 was obtained from Pharmacia Fine Chemicals. Oxo was a gift of Warner-Lambert Research Institute. ϕ X174 DNA (7), ϕ X174 RFI (2), and ColE1 DNA (8) were prepared as described. Relaxed ϕ X174 RF and ColE1 DNA were prepared either by nicking with pancreatic DNase followed by sealing with T4 DNA ligase (9) or by relaxation with *E. coli* ω protein (6).

Enzyme Assays. The Pnal assay, which will be detailed elsewhere, was essentially that devised by C. Sumida-Yasumoto using a *nalA*^r strain constructed by R. Sternglanz. It is based on the dominant conferral of drug sensitivity by addition of wild-type protein to a ϕ X174 RFI replication system (2) directed by H560-1 extracts. The reaction mixtures (0.05 ml) contained 1 nmol of ϕ X174 RFI, 0.1 mg of H560-1 receptor ($(\text{NH}_4)_2\text{SO}_4$ fraction, 10 μ g of Fraction II prepared from H560 infected with ϕ X174 *am3* Pnal, and 30 μ g/ml of Oxo or 100 μ g/ml of Nal. One unit of Pnal catalyzes the sensitization of 1 nmol of dTMP incorporation to Nal or Oxo in 30 min at 30°.

The DNA gyrase assay measures the conversion of relaxed ColE1 DNA to the supercoiled form as monitored by agarose gel electrophoresis (3). The reaction mixture (35 μ l) contained 35 mM Tris-HCl at pH 7.6, 6 mM MgCl_2 , 18 mM potassium phosphate, 5 mM spermidine-HCl, 1.4 mM ATP, yeast tRNA at 90 μ g/ml, 5 mM dithiothreitol, bovine serum albumin at 50 μ g/ml, 0.2 μ g of relaxed DNA, and enzyme. After 60 min at 30°, 10 μ l of a 25% (vol/vol) glycerol solution containing bromophenol blue at 0.25 mg/ml and either 5% sodium dodecyl sulfate (NaDodSO_4), 2.5% Sarkosyl, or 45 mM EDTA was added. The mixture was applied to a 13 $\times$ 15 $\times$ 0.4 cm slab gel of 1.0% agarose and then subjected to electrophoresis at 40 V for 14-16 hr at 23° (3). The gels were stained, destained, and photographed using shortwave ultraviolet light (3). Negatives were traced with a Joyce-Loebl microdensitometer. One unit of DNA gyrase converts 0.1 μ g of relaxed DNA to the super-

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Abbreviations: Nal, nalidixic acid; Oxo, oxolinic acid; Pnal, Nal target protein; N-C, nicking-closing; RF, replicative form; NaDodSO_4 , sodium dodecyl sulfate. An allele conferring resistance to a drug is indicated by ^r.

Table 1. Purification of Pnal

Fraction and step	Activity, units	Protein, mg	Specific activity, units/mg
I Crude extract	—	18,000	—
II (NH ₄) ₂ SO ₄	8,900	5,000	1.8
III DEAE-cellulose	5,500	300	18
IV Hydroxylapatite	4,400	32	140
V Phosphocellulose	2,200	0.22	10,000
VI Sephadex G-200	1,240	0.108	11,500

H560 cells (260 g) were lysed as described (7). Subsequent buffers contained 10 mM 2-mercaptoethanol, 1 mM EDTA, and 10% glycerol except where indicated. After centrifugation of the extract for 60 min at $170,000 \times g$, the supernatant (300 ml, Fraction I) was made to 4% streptomycin sulfate and centrifuged at $27,000 \times g$ for 10 min. The supernatant was made 45% saturated with (NH₄)₂SO₄ and the mixture was centrifuged at $27,000 \times g$ for 15 min. The pellet was resuspended in 40 ml of 50 mM Tris-HCl, pH 7.5, dialyzed against this buffer for 3 hr (Fraction II, 50 ml), and applied to a DE52 column (5.6 $\times$ 35 cm) equilibrated with dialysis buffer containing 25 mM NaCl. After a 1.5-liter buffer wash, the column was developed with a 4-liter linear gradient of 0.025–0.5 M NaCl in 50 mM Tris-HCl, pH 7.5. Active fractions (0.12–0.2 M NaCl) were pooled (400 ml) and precipitated with 128 g of (NH₄)₂SO₄. The pellet after centrifugation was dissolved in 10 ml of 20 mM potassium phosphate, pH 6.8 (without EDTA), dialyzed against the buffer for 4 hr (Fraction III), and applied to a hydroxylapatite column (2.05 $\times$ 31 cm) equilibrated with the same buffer. After a 70-ml buffer wash, the column was developed with a 0.02–0.5 M potassium phosphate, pH 6.8, gradient containing 10 mM 2-mercaptoethanol and 10% glycerol. Active fractions (0.1–0.2 M phosphate) were concentrated to 10 ml by dialysis against polyethylene glycol and dialyzed against 25 mM phosphate, pH 6.8 (Fraction IV). The protein was applied to a P11 column (1.6 $\times$ 31 cm) equilibrated with the dialysis buffer. After a 60-ml buffer wash, protein was eluted with a 240-ml gradient of 0.025–0.5 M phosphate, pH 6.8. Active fractions (0.07–0.15 M phosphate) were concentrated as above and dialyzed against 0.2 M phosphate, pH 7.4 (Fraction V, 5 ml). This sample was fractionated on a Sephadex G-200 column (1.75 $\times$ 40 cm) equilibrated with the same buffer. Active fractions (partition coefficient K_d of 0.1–0.2) were concentrated and dialyzed against 50 mM Tris-HCl at pH 7.5 and 50% glycerol.

coiled form in 30 min at 30°. The assay for nicking-closing (N-C) was the same as for DNA gyrase except ColE1 or ϕ X174 RFI DNA was the substrate and ATP was omitted.

Enzymes. Proteinase K was obtained from EM Laboratories. Homogeneous *E. coli* ω protein and antisera to it were provided by R. Depew and J. Wang. The purification of Pnal is summarized in Table 1. Wild-type DNA gyrase was purified simultaneously with Pnal. In step IV, gyrase eluted from 0.2 M to 0.3 M phosphate. The enzyme was then purified by phosphocellulose chromatography as described for Pnal—the gyrase eluted broadly from 0.1 to 0.25 M phosphate. This fraction (about 6000 units/mg of protein) was used after concentration and had no detectable DNA endonuclease or exonuclease activity under standard assay conditions, but had *nalA* complementing activity (450 units/mg of protein). DNA gyrase from *E. coli* H560-1 cells (1100 units/mg) was purified similarly except the phosphocellulose step was omitted.

Determination of the Molecular Weight of Pnal. Native molecular weight was calculated (10) from the sedimentation rate through a 35–50% glycerol gradient and the elution from a Sephadex G-200 column measured in 0.2 M phosphate, pH 7.4/10 mM 2-mercaptoethanol/1 mM EDTA. NaDodSO₄ containing polyacrylamide gel electrophoresis used 7% (gel I) and 11% (gel II) gels prepared according to Laemmli (11) and 10% gels (gel III) prepared according to Anderson *et al.* (12).

Isolation of λ Transducing Phages. Secondary site lysogens (13) with λ c1857 inserted into *glpT* were selected by fosfomycin

FIG. 1. NaDodSO₄ gel electrophoresis of *nalA* gene product. In A, 4 μ g of Fraction VI Pnal was electrophoresed through gel I and the protein was stained with Coomassie blue. In B, gel III was used. Lane a contains T4 proteins labeled with [³⁵S]sulfate from 18 to 23 min after infection at 37° (14); lane b, Pnal protein; lanes c and d, proteins containing ¹⁴C-labeled amino acids from λ dnaA- and λ dubiG-infected, ultraviolet light-irradiated λ lysogens, respectively. The center of the stained Pnal band was marked with a dot of ¹⁴C, and the gel was fluorographed.

resistance. Induction of the lysogens produced phages that transduced both *nalA* and *ubiG* (λ dnaA) and phages that transduced *ubiG* but not *nalA* (λ dubiG).

RESULTS

Purification of Pnal. The assay used for purification measures conferral of Nal or Oxo sensitivity to ϕ X174 RFI replication directed by enzymes from *nalA*⁺ cells by the addition of wild-type protein. By the procedure described in Table 1, Pnal was purified to homogeneity as monitored by NaDodSO₄/polyacrylamide gel electrophoresis (Fig. 1A). A single polypeptide was found of molecular weight 110,000. It has a mobility identical to *E. coli* ω protein. The Pnal preparation was free of detectable endonuclease, DNA ligase, and DNA polymerase. It contained a trace of DNA gyrase activity (150 units/mg of protein). The ratio of *nalA*-complementing activity to gyrase activity increased throughout purification and was about 1000-fold greater in Fraction VI than in Fraction II.

To prove that Pnal is a *nalA* gene product, λ transducing phages were constructed that contained either both *nalA* and the tightly linked *ubiG* (λ dnaA) or *ubiG* but not *nalA* (λ dubiG). An ultraviolet light-irradiated λ *ind*[−] lysogen was infected by the transducing phages and the proteins synthesized were labeled with ¹⁴C-labeled amino acids (15). Of the 11 polypeptides induced by λ dnaA resolved by electrophoresis through gel II, only one, with a molecular weight of 110,000, was not found after infection by λ dubiG. The experiment was repeated with gel III to expand the high molecular weight region (Fig. 1B). The peptide unique to λ dnaA (lane c) has the same mobility as a Pnal standard marked with a dot in lane b. We conclude that purified Pnal is a product of the *nalA* gene.

The molecular weight of native Pnal was estimated from the sedimentation coefficient and Stokes radius (10). A single peak

FIG. 2. Inhibition of DNA gyrase by Oxo and Nal and substrate cleavage. Standard DNA gyrase reactions with 1 unit of enzyme contained either Fraction IV gyrase from strain H560-1 (closed symbols) or H560 enzyme (open symbols, Fraction V in A and Fraction IV in B and C) and the indicated concentration of Oxo (circles) or Nal (squares). In C, supertwisted ColE1 DNA replaced the relaxed substrate. The reactions were stopped with EDTA in A and NaDodSO₄ in B and C. After agarose gel electrophoresis, the gels were stained and scanned, and the amounts of product (supertwisted DNA in A and B and linear molecules in C) were calculated. v_0 is the reaction rate (v) in the absence of drug. The concentrations of drug causing 50% inhibition are, respectively, 10 and 5 $\mu\text{g/ml}$ for Oxo in A and B for H560 enzyme and 1000 and 250 $\mu\text{g/ml}$ for Oxo in A and B for H560-1 enzyme, and 200 and >2000 $\mu\text{g/ml}$ for Nal for H560 and H560-1 enzyme.

of *nalA*-complementing activity was observed after both sedimentation through a 35–50% glycerol gradient and filtration through Sephadex G-200. The Stokes radius and $s_{20,w}$ of 55 Å and 10.6 S for Pnal, taken with an assumed partial specific volume of 0.725, lead to a molecular weight of 240,000. This value is consistent with Pnal being a dimer of identical 110,000-dalton subunits.

Inhibition of DNA Gyrase by Nal and Oxo. DNA gyrase was extensively purified from wild-type and *nalA*^r cells; however, neither preparation was homogeneous. The wild-type gyrase preparation contained *nalA* complementing activity; it is unknown if it is intrinsic to the enzyme. The gyrase from wild-type cells was strongly inhibited by both Nal and Oxo; 10 $\mu\text{g/ml}$ of Oxo halved supertwisting activity (Fig. 2A). The gyrase from the *nalA*^r mutant was two orders of magnitude less drug sensitive (Fig. 2A). The linearity of the Dixon plots implies that inhibition is kinetically simple. Oxo was 20 times more effective than Nal (Fig. 2A), which is about the same potency ratio as found *in vivo* (1). Wild-type and mutant enzyme preparations were equally sensitive to novobiocin. We conclude that DNA gyrase activity is sensitive to Nal and Oxo and that inhibition requires the *nalA* gene product.

Nal and Oxo Promote a Relaxation-Type Complex with DNA Gyrase and Its Substrate. The addition of Nal or Oxo does not simply prevent catalysis by DNA gyrase. When the reaction products were treated with NaDodSO₄, there was some

FIG. 3. DNA cleavage by DNA gyrase induced by Oxo. Standard DNA gyrase reaction mixtures, except i and j did not have ATP, contained: no enzyme in a and 1 unit of H560 gyrase in b–n; native ColE1 DNA in a–m, and ϕ X174 RFI in n; Oxo at 28 $\mu\text{g/ml}$ in all except j and novobiocin at 150 $\mu\text{g/ml}$ in d. After 60 min at 30°, *Eco*RI-restricted ColE1 DNA was added to k–m, and the NaDodSO₄ stop solution was added to a, c, d, i, j, and l–n; the Sarkosyl stop solution, to b and k; and only 10 mM EDTA (final concentration) to e–h. Samples e–h and m were further treated by: heating at 65° for 10 min for e; heating, then adding NaDodSO₄ to 1% for f; digesting with proteinase K at 100 $\mu\text{g/ml}$ for 60 min at 37° for g and m; and digesting with proteinase K followed by adding NaDodSO₄ for h. The products were analyzed by agarose gel electrophoresis. The prominent bands are relaxed plus nicked DNA, linear DNA, and supertwisted DNA, in order of increasing mobility (top to bottom).

smearing of the agarose gel patterns and a new band appeared that migrated slightly more slowly than linear DNA. The linear structure of the product was confirmed by electron microscopy—the fraction of linear molecules increased from < 1% to 41% following NaDodSO₄ treatment. Activation of DNA cleavage by a protein denaturing agent also occurs with relaxation complexes (16) and presumed intermediates in the ω protein reaction (17). The Oxo- or Nal-induced cleavage has the following properties: First, the reaction shows genetic specificity. Half-maximal cleavage required 5–10 $\mu\text{g/ml}$ of Oxo with wild-type DNA gyrase and two orders of magnitude more drug with gyrase from *nalA*^r cells, the same dose response as observed for inhibition of supertwisting (Fig. 2B and C). The two-order-of-magnitude difference in Oxo sensitivity of wild-type and mutant gyrase in the cleavage and supertwisting reactions was observed at all stages of enzyme purification. Second, treatment of the reaction products with 10 mM EDTA, 0.5% Sarkosyl, proteinase K (100 $\mu\text{g/ml}$ for 60 min at 37°), or heat (65° for 10 min) did not cleave the substrate (Fig. 3 b, e, and g). Pretreatment with proteinase K or heating at 65° for 10 min prevented NaDodSO₄-induced cleavage (Fig. 3 f and h), which implies NaDodSO₄ activation of a nuclease rather than inactivation of a protein linker in the DNA (cf. ref. 16). Third, relaxed and supertwisted circular DNA (Fig. 3 c and n; Fig. 4k) and linear DNA were cleaved. Fourth, unlike supertwist introduction, cleavage did not require ATP and was novobiocin resistant (Fig. 3 d, i, and j). With novobiocin at 150 $\mu\text{g/ml}$, 100 times the concentration that effectively inhibits supertwisting, there was little or no inhibition of the cutting of supertwisted and relaxed substrates. Fifth, the cleaved DNA is probably covalently linked to a protein. One-third of the product is less dense than the substrate as determined by equilibrium density centrifugation; the appearance of the light material requires enzyme, drug, and NaDodSO₄ treatment and is prevented by Pronase digestion. Also, the cleaved ColE1 DNA product migrates more slowly on agarose gels than *Eco*RI-cut ColE1 DNA but after proteinase K digestion they have the same mobility (Fig. 3 k–m). Sixth, cleavage, like supertwist introduction, requires Mg^{2+} and is inhibited by *N*-ethylmaleimide. Seventh, specific cleavage sites were found in ColE1, mini ColE1, λ , and T7 DNA.

Effect of Pnal on DNA Gyrase. Wild-type Pnal has two clear effects on DNA gyrase. First, Pnal stimulated DNA gyrase

FIG. 4. Wild-type Pnal stimulates DNA gyrase and complements DNA gyrase from *nalA*^r cells. Standard-gyrase reaction mixtures contained: no enzyme in a; 0.1 unit of H560 Fraction V DNA gyrase in b–d and also 0.1 and 0.6 unit of Pnal in c and d, respectively; 0.6 unit of Pnal in e; 0.5 unit of H560-1 Fraction IV gyrase in f–k and also 0.1 unit of Pnal in g and j and 0.6 unit of Pnal in h and k; and Oxo at 57 $\mu\text{g}/\text{ml}$ in i–k. After the reaction had been stopped with 1.0% NaDodSO₄, the products were displayed by agarose gel electrophoresis. The scan of the stained gels is shown and the origin is at the left.

supercoiling activity. The degree of stimulation is a function of the DNA gyrase level but was as large as an order of magnitude (Fig. 4 a–d). At the level used, Pnal alone showed no detectable supercoiling activity (Fig. 4e) or drug-induced DNA cleavage. Second, wild-type Pnal conferred Nal and Oxo sensitivity to DNA gyrase from *nalA*^r cells. Both the inhibition of supercoiling and the cleavage of DNA resulting from drug addition were complemented (Fig. 4 f–k). These experiments show an intimate relationship between Pnal and gyrase.

Identification of an Oxo- and Nal-Sensitive Nicking-Closing Enzyme and Comparison with ω Protein. Because DNA gyrase alters the supercoiling density of DNA, it is expected to have an N-C component. This activity was found after addition of novobiocin to gyrase reactions with supercoiled ColE1 DNA or ϕX174 RFI substrates (Fig. 5 c and e). Novo-

biocin is needed only to inhibit supercoiling because omission of ATP also revealed relaxation activity (Fig. 5f). Two direct pieces of evidence relate N-C activity to the *nalA* gene product and thereby also to DNA gyrase. First, Pnal had N-C activity (Fig. 5 r and u) with the same properties as that in DNA gyrase. Second, the N-C activity in gyrase from wild-type (Fig. 5g) but not *nalA*^r cells was inhibited by Nal or Oxo. Half-maximal inhibition of N-C activity in both Pnal and wild-type gyrase required Oxo at 5–10 $\mu\text{g}/\text{ml}$ —the same sensitivity as inhibition of supercoiling and substrate cleavage. Because Pnal, which has negligible gyrase activity, has N-C activity, the N-C enzyme can act independently as well as in its proposed role as a component of supercoiling by gyrase. The ratio of N-C to supercoiling activity in different gyrase preparations varied widely.

The only *E. coli* enzyme described previously that relaxes DNA is ω protein (6). The activity that we have discovered and designate as N-C enzyme is distinct from ω by several criteria. First, while 0.1 μl of antibodies directed against ω inhibited ω activity completely, up to 3 μl did not affect N-C enzyme (Fig. 5 h–j) or supercoiling introduction. Second, N-C enzyme but not ω relaxed positively supercoiled DNA (Fig. 5 s–v). Third, ω was only slightly affected by Oxo at 85 $\mu\text{g}/\text{ml}$, a concentration that inhibited N-C enzyme completely (Fig. 5 g and k). Fourth, 0.35 nmol (total nucleotide) of ϕX174 single-stranded DNA inhibited ω completely but even 2.8 nmol of ϕX174 DNA did not affect N-C enzyme (Fig. 5 l and m). The possibility that something in the N-C enzyme preparation prevented inhibition by antibodies and single-stranded DNA and promoted inhibition by Oxo was ruled out by mixed extract experiments (Fig. 5 n–q). Note also that ω acted less processively to give a narrower supercoiling density distribution at low enzyme levels.

DISCUSSION

Elucidating the mechanism of a drug requires pure target protein. In this paper we report the extensive purification of a target protein, using only a complementation assay based on drug sensitivity. This should be a generally valuable technique for studying inhibitors and DNA metabolism target enzymes when the catalytic activity of the target is unknown. Pnal is probably a dimer of identical 110,000-dalton subunits. The comigration in NaDodSO₄/polyacrylamide gels of Pnal and

FIG. 5. Comparison of N-C enzyme and ω protein. Standard N-C reaction mixtures were used except in d and e the substrate was ϕX174 RFI, a–e contained 1.4 mM ATP, and s–v contained positively supercoiled ColE1 DNA made (8) by ligating nicked DNA at 4° and including in the reaction 0.2 μM ethidium bromide; the dye was removed before gel analysis. Assays contained no enzyme in a, d, and s; 1 unit of H560 Fraction V DNA gyrase in b, c, e–g, j, m, and t; 100 units (9) of ω protein in h, i, k, l, and v; ω and gyrase in n–q; 5 units of Pnal in r and u; novobiocin at 143 $\mu\text{g}/\text{ml}$ in a and c–e; Oxo at 86 $\mu\text{g}/\text{ml}$ in g, k, and q; antisera to ω (0.1 μl in i and 3 μl in j and o); and ϕX174 single-stranded DNA (in total nucleotide, 0.35 nmol in l, 2.8 nmol in m, and 0.7 nmol in p). The reaction products were displayed by agarose gel electrophoresis. The high-mobility bands in l, m, and p are ϕX174 single-stranded DNA.

the only labeled polypeptide band induced uniquely by λ dnalA provides strong evidence that Pnal is a *nalA* gene product.

The *nalA* gene product can be intimately associated with DNA gyrase. First, Oxo and Nal inhibit the wild-type enzyme markedly but DNA gyrase from *nalA*^r cells is 100 times more resistant. The order-of-magnitude greater potency of Oxo compared to Nal also accords with other *in vivo* and *in vitro* data (1). Second, wild-type Pnal stimulates DNA gyrase activity and renders the activity of gyrase from *nalA*^r cells drug sensitive. Third, wild-type DNA gyrase preparations contain *nalA*-complementing activity and, like Pnal, N-C activity.

There are two extreme models for the association of the *nalA* gene product and DNA gyrase. The *nalA* gene product could be an essential subunit of DNA gyrase, as is presumably the *cou* gene product (5). If, in addition, the subunits are in equilibrium with the complete proteins, Pnal stimulation of gyrase activity and complementation of resistant gyrase are readily explained. Alternatively, the association could be adventitious and wild-type *nalA* gene product in the presence of drug could alter the DNA substrate or DNA gyrase so as to prevent supercoiling and induce cleavage after NaDodSO₄ treatment.

The three reactions catalyzed by DNA gyrase preparations that are affected by Nal and Oxo are: (i) introduction of negative supertwists, (ii) cleavage of DNA after NaDodSO₄ treatment, and (iii) relaxation of DNA. The strong evidence that *nalA* gene product is required for the drug effects on these reactions is the drug resistance of gyrase from *nalA*^r cells and, for reactions i and ii, the complementation of this enzyme by wild-type Pnal. The N-C activity of the highly purified Pnal provides independent evidence for this conclusion for the third reaction. By definition, reaction i is catalyzed by DNA gyrase. The evidence is very good that reaction ii is intrinsic to DNA gyrase but is less direct for reaction iii. Four DNA gyrase preparations extensively purified by three different procedures carried out reactions i and ii with about equal relative efficiency. All three reactions are N-ethylmaleimide-sensitive, require Mg²⁺, are resistant to inhibition by single-stranded DNA, and are similarly inhibited by high salt concentrations.

The apparently complex pattern of interactions of at least two proteins with three different drugs can be explained by a simple model. Consider DNA gyrase to be made up of two components—an N-C enzyme and a DNA-melting (or equivalent) enzyme. The relaxation by the N-C component of the positive supertwists introduced by melting a region of closed circular DNA followed by renaturation of the melted region leads to negative supertwisting. The degree of independence of the gyrase components could vary between the extremes of subunits of a single multimeric protein to discrete but interacting enzymes; however, the independence, *in vitro*, is sufficiently great that N-C activity can be expressed independent of the melting component and inhibition of one component need not affect the other. An N-C enzyme must be involved in supertwisting DNA if the helical parameters are undisturbed; the melting component is a means of making the supertwisting change vectorial toward the higher-energy, underwound state. By analogy with other such enzymes, the N-C activity need not require energy (9, 18) but melting of the duplex does (reviewed in ref. 19), and thus this is the reaction that requires ATP. We postulate that the N-C and melting activities are coded by the *nalA* and *cou* genes, respectively, or, at least, are functionally and intimately associated with the gene products. The model readily explains the sensitivity of the three reactions catalyzed

by DNA gyrase to Oxo and Nal, the resistance of reactions ii and iii to novobiocin, the ATP requirement of only reaction i, the N-C and *nalA*-complementing activity of Pnal and DNA gyrase, and the interaction of Pnal and DNA gyrase. NaDodSO₄- and Oxo- or Nal-induced substrate cleavage is directly analogous to the NaDodSO₄-induced cleavage of presumed intermediates in the reaction of another topoisomerase, ω protein (17). In both cases, the protein-linked product is logically a reflection of the high-energy enzyme-substrate complex that is competent for resealing (6). A derivative of this intermediate is then revealed by the Nal or Oxo inhibition of the gyrase reaction. Since we observe double-strand breaks, the gyrase intermediate may have a unique structure in which enzyme is bound to both DNA strands. The N-C enzyme we have discovered is distinct from ω . The two enzymes respond differently to antibodies against ω , Oxo, Nal, single-stranded DNA, N-ethylmaleimide, and Mg²⁺ concentration. Significantly, only N-C enzyme relaxes positive supertwists, which is a desirable feature for an enzyme involved in maintaining supertwist tension or which acts as a swivel for DNA replication and previously was known to be a property only of eukaryotic relaxing enzymes (18).

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