

DNA-relaxing enzyme from *Micrococcus luteus*

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ABSTRACT

A DNA-relaxing enzyme which catalyzes the conversion of superhelical DNA to a non-superhelical covalently closed form has been purified from *Micrococcus luteus* to near homogeneity by two chromatographic steps. The enzyme is a single polypeptide chain. As determined by sodium dodecyl sulfate - polyacrylamide gel electrophoresis and gel filtration on Sephadex G 150, the molecular weight is 115,000. The DNA-relaxing activity determined as a function of enzyme concentration follows a sigmoidal curve. The enzyme requires  $Mg^{++}$  for activity. In the presence of 4.5 mM  $Mg^{++}$  addition of 50-250 mM KCl yields incompletely relaxed DNA molecules (intermediates); intermediates are also observed in the absence of KCl, when the reaction is carried out at 0°C or at  $Mg^{++}$  concentrations exceeding 10 mM.

INTRODUCTION

DNA-relaxing enzymes catalyze the conversion of superhelical DNA to a non-superhelical covalently closed form (1-4) presumably by introducing a transient swivel into the helix (5). The relaxation process probably does not follow a single-hit mechanism as intermediates are generated having smaller numbers of superhelical turns than the original DNA substrate (3,5,6). It seems accepted that the essential steps of the DNA relaxation involve breakage of one strand, winding of that strand relative to the other and sealing of the break (5,7). The *in vivo* functions of DNA-relaxing enzymes are still uncertain. The enzymes may be involved in any process requiring winding or unwinding of the double helix such as replication (8) or transcription (9). DNA-relaxing activities have been purified from *Escherichia coli* (1,10), vaccinia virus (11), *Drosophila melanogaster* (3), yeast (Thielmann and Hess, in preparation), calf thymus (12), rat liver (13), mouse embryo cells (4), HeLa (4) and KB cells (6). The enzymes of eukaryotic origin are distinguished from the  $\omega$  protein of *E.coli* in that they relax both negatively and positively twisted DNAs and they do not require  $Mg^{++}$  for activity (1-4,6,12,13). In this communication we report the purification and preliminary characterization of a DNA-relaxing enzyme from *M.luteus*.

### MATERIALS AND METHODS

Assay of DNA-relaxing activity. The DNA-relaxing activity was assayed following the method described by Keller (6). The standard incubation mixture (total volume 20  $\mu$ l) contained 50 mM Tris-HCl pH 7.4, 4.5 mM MgCl<sub>2</sub>, 0.3 nmol PM2 DNA (nucleotides) and DNA-relaxing enzyme dissolved in 5  $\mu$ l 10 mM potassium phosphate pH 7.3, 50% glycerol. PM2 DNA had been prepared according to Espejo et al. (14). The reaction proceeded for 30 min at 37°C and was then stopped with 10  $\mu$ l of 10% sodium dodecyl sulfate. After addition of 5  $\mu$ l of 0.04% bromophenol blue marker and 10  $\mu$ l 87% glycerol, the DNA was analyzed by agarose gel electrophoresis.

Gel electrophoresis. Vertical slab gels (20 x 15 x 0.3 cm) were formed between rough surface glass plates using 1% agarose (Seakem) dissolved at 100°C in electrophoresis buffer (40 mM Tris-HCl pH 7.5, 5mM sodium acetate, 1 mM EDTA, 5 mM MgCl<sub>2</sub>). Gels and buffer contained Mg<sup>++</sup> to separate the limit product of the DNA relaxation process (form I<sup>0</sup>) from the nicked form (product of possible concomitant endonuclease activity). Mg<sup>++</sup> imposes negative superhelical turns to covalently closed circular DNA thus causing increased migration velocity in gel electrophoresis relative to the nicked form (15). DNA samples were run for 12 h at constant voltage (60 V) at room temperature. Gels were stained with ethidium bromide (2  $\mu$ g/ml electrophoresis buffer) for 1 h in the dark, illuminated from below with ultraviolet light (transilluminator emitting predominantly 253.7 nm radiation, Ultra-Violet Products, Inc., San Gabriel, Calif., USA) and photographed on Polaroid type 107 films. To obtain the relative quantities of DNA represented by the intensity of the respective bands, photographs were traced with a Joyce-Loebl microdensitometer. The peak areas were cut out and weighed.

One unit of DNA-relaxing activity is defined as the amount of enzyme that converts 0.15 nmol of superhelical PM2 DNA into the completely relaxed form I<sup>0</sup> under the standard conditions described above.

Purification of the DNA-relaxing enzyme. Unless otherwise indicated, all steps were performed at 4°C. Frozen M.luteus cells (125 g wet weight; Serva, Heidelberg) were suspended in 560 ml of 10 mM potassium phosphate pH 7.3, 5 mM  $\beta$ -mercaptoethanol, 1 mM EDTA (buffer A) and thawed. Lysis of the cells was achieved by incubation with lysozyme (final concentration 320  $\mu$ g/ml) for 30 min at 37°C with continuous stirring. The resulting viscous material was chilled to 0°C and sonified with a Branson sonifier (model S 125), until all the viscosity had disappeared. The sonicated solution was centrifuged at 23,000 x g for 40 min. Dextran T 500 (63 ml of 20% w/w in

buffer A), polyethylene glycol 6,000 (177 ml of 30% w/w in buffer A) and 93 g solid NaCl were added to the supernatant (550 ml, fraction I). After stirring for 2 h, phase separation was achieved by centrifugation at 5,000 x g for 15 min. The clear upper phase was dialyzed against 50 mM potassium phosphate pH 7.3, 5 mM  $\beta$ -mercaptoethanol, 1 mM EDTA, 10% ethylene glycol (buffer B). A precipitate resulting from dialysis was removed by centrifugation at 30,000 x g for 10 min; 750 ml of supernatant were obtained (fraction II). Fraction II was passed through a phosphocellulose column (4 x 8.5 cm) pre-equilibrated with buffer B, followed by a linear gradient (600 ml) of 0.05 - 0.5 M potassium phosphate pH 7.3, 5 mM  $\beta$ -mercaptoethanol, 1 mM EDTA, 10% ethylene glycol. Fractions of 6.3 ml were collected and assayed. Active fractions were combined (fraction III), dialyzed against 50 mM Tris·HCl pH 7.5, 5 mM  $\beta$ -mercaptoethanol, 1 mM EDTA, 10% ethylene glycol and applied to a column (3 x 5.3 cm) of UV-irradiated double-stranded DNA-cellulose pre-equilibrated with the same buffer. The DNA-cellulose had been prepared according to Litman (16). The column was washed with two volumes of equilibration buffer, followed by a linear gradient (166 ml) of 0.15 - 2.5 M NaCl in the same buffer. Fractions of 3.2 ml were collected, dialyzed against buffer B and tested. Enzyme containing fractions were stored with 50% glycerol at -20°C (Fraction IV). Under these storage conditions, the enzyme was stable for at least six months.

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis. Proteins were reduced and denatured by heating for 10 min at 95°C in the presence of 1%  $\beta$ -mercaptoethanol and 1% sodium dodecyl sulfate, and then subjected to disc electrophoresis in the presence of 0.03% sodium dodecyl sulfate (17); the separating gels contained 8% acrylamide and 0.32% bisacrylamide. The gels were stained overnight in 0.035% Comassie brilliant blue in 10% acetic acid, 25% propanol-(2), and destained in 10% acetic acid. The positions of the protein bands in the gels were determined relative to bromophenol blue.

Sephadex G 150 chromatography. For determination of molecular weight of the DNA-relaxing enzyme, an aliquot of fraction III was loaded on a Sephadex G 150 column (1.6 x 25 cm) equilibrated with buffer B and calibrated with marker proteins. The enzyme was eluted with buffer B; fractions of 1 ml were collected.

## RESULTS

Purification of the DNA-relaxing enzyme. M.luteus cells were lysed by lysozyme treatment followed by sonification. DNA was removed by dex-

tran-polyethylene glycol phase partition. Two chromatographic steps sufficed to purify the DNA-relaxing enzyme to near homogeneity. The first step involved ion exchange chromatography on phosphocellulose; the enzyme eluted between 0.2 - 0.4 M potassium phosphate (Fig. 1). The second step involved chromatography of the active fractions from the phosphocellulose column on UV-irradiated double-stranded DNA-cellulose. As Fig. 2 shows, the enzyme bound very strongly to the column material, since elution required 1.8 to

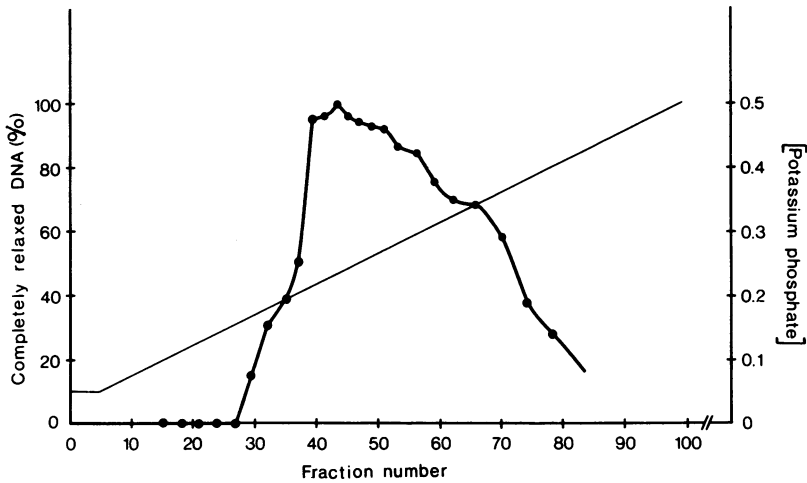


Fig. 1. Elution of the DNA-relaxing enzyme from phosphocellulose. Fractions were diluted 1:30 and assayed under standard conditions as described in Materials and Methods. (●—●) DNA-relaxing activity; (—) potassium phosphate concentration.

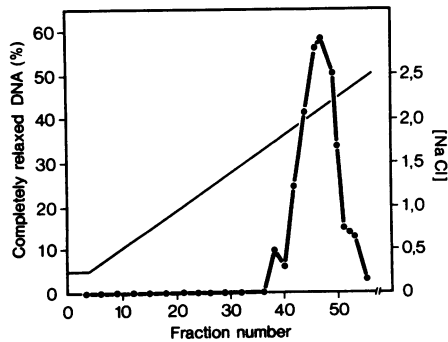


Fig. 2. Elution of the DNA-relaxing enzyme from UV-irradiated double-stranded DNA-cellulose. Fractions were diluted 1:10 and assayed under standard conditions as described in Materials and Methods. (●—●) DNA-relaxing activity; (—) NaCl concentration.

2.2 M NaCl. By the procedure summarized in Table 1, 160  $\mu$ g of homogeneous DNA-relaxing enzyme were obtained from 125 g of frozen cells. Assaying the DNA-relaxing activity was not impeded or masked by endonucleases at any stage of purification.

Table 1. Purification of the DNA-relaxing enzyme from *M.luteus*. DNA-relaxing activity was measured as described in Materials and Methods; protein was determined according to Lowry et al. (18)

Fraction	Total activity (units $\times 10^{-6}$ )	Yield (%)	Specific activity (units/mg protein $\times 10^{-4}$ )	Purification (-fold)
I (crude extract)	27.5	100	1.1	1
II (polyethylene glycol phase)	18.6	68	2.3	2.1
III (phosphocellulose column fractions)	5.1	19	6.6	6
IV (DNA-cellulose column fractions)	0.22	1	148	135

On phosphocellulose, two repair endonucleases co-chromatographed with the DNA-relaxing enzyme; one acting on UV-irradiated DNA (19) and a second one specific for apurinic sites. Separation of the DNA-relaxing enzyme from these endonucleolytic activities occurred during the chromatography on UV-irradiated DNA-cellulose. Purification of the repair endonucleases will be published elsewhere.

Purity and molecular weight. Sodium dodecyl sulfate - polyacrylamide gel electrophoresis of the purified enzyme (fraction IV) revealed only a single protein band (Fig. 3) with a mobility corresponding to a molecular weight of  $118,000 \pm 12,000$  daltons (Fig. 4a). The experiment depicted in Fig. 4a is representative for four independent electrophoretic runs. When the particular column fractions (fraction IV) were assayed for both, homogeneity (sodium dodecyl sulfate - polyacrylamide gel electrophoresis) and DNA-relaxing activity, the intensity of the 118,000 protein band correlated with the enzyme activity (data not shown). Using gel filtration on Sephadex G 150, the molecular weight of the DNA-relaxing enzyme was found to be  $112,000 \pm 8,000$  daltons (Fig. 4b).



Fig. 3. Densitometer tracing of a sodium dodecyl sulfate - polyacrylamide gel of purified DNA-relaxing activity. 2,000 units DNA-relaxing activity from fraction IV were subjected to disc electrophoresis as described in Materials and Methods. The gel was scanned at 600 nm with a Gilford recording spectrophotometer equipped with a linear transport device.

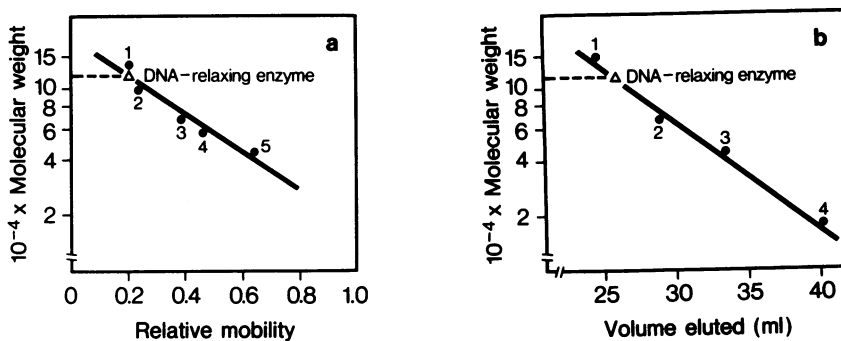


Fig. 4. Molecular weight determinations of the DNA-relaxing enzyme by (a) sodium dodecyl sulfate - polycrylamide gel electrophoresis and (b) gel filtration on Sephadex G 150. a) DNA-relaxing activity from fraction IV and marker proteins were subjected to disc electrophoresis as described in Materials and Methods; (1)  $\beta$ -galactosidase, 130,000; (2) phosphorylase a, 100,000; (3) bovine serum albumin, 68,000; (4) catalase, 58,000; (5) ovalbumin, 43,000. b) An aliquot of fraction III was chromatographed on Sephadex G 150 as described in Materials and Methods. Marker proteins: (1) aldolase, 147,000; (2) bovine serum albumin, 68,000; (3) ovalbumin, 43,000; (4) myoglobin, 17,200.

Dependence of enzyme activity on enzyme concentration. The DNA-relaxing activity determined as a function of enzyme concentration followed a sigmoidal curve (Fig. 5). Without having as yet the additional experimental evidence needed for thorough interpretation of the observed kinetics, it seems reasonable to assume that the DNA-relaxing enzyme attains its full activity as a multimer and that at very low enzyme concentrations, formation of the multimeric complex becomes rate limiting. It is unlikely that enhanced

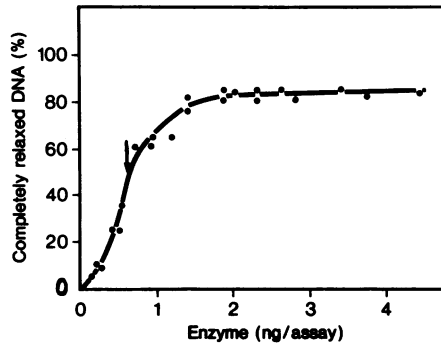


Fig. 5. DNA-relaxing activity as a function of enzyme concentration. DNA-relaxing activity (fraction IV) was assayed under standard conditions as described in Materials and Methods.

enzyme denaturation accounts for the decrease in activity, since sigmoidal curves were also obtained with the less purified fractions I-III (data not shown).

From Fig. 5 it can also be deduced that 8.3 fmol PM2 DNA (mol. weight:  $6 \times 10^6$ , 18,200 nucleotides (14)) were relaxed by 5.6 fmol enzyme (see arrow). Taking into account that the relaxation process apparently follows a multi-hit mechanism and that one PM2 DNA molecule has 100 superhelical turns (20-22), one enzyme molecule removed approximately 150 superhelical turns under standard conditions. Therefore, we conclude that the DNA-relaxing enzyme from *Micrococcus luteus* acts in a catalytic way as do other DNA-relaxing enzymes (5,6,11,13,23).

Enzyme activity in the presence of potassium chloride or at 0°C. Fig. 6a, channel 2 shows that in the presence of 4.5 mM  $Mg^{++}$  but in the absence of KCl, the relaxation of PM2 DNA was complete; addition of less enzyme resulted in complete relaxation of less DNA molecules (Fig. 6b and c, channels 2). With increasing concentrations of KCl (channels 3-11), however, sets of intermediates were formed, which migrated faster in gel electrophoresis than the original limit product, form I<sup>0</sup>. Obviously, addition of KCl slowed down the relaxation process, yielding DiA species with higher superhelical winding numbers. Coincidentally, for a given enzyme concentration, the fraction of DNA molecules which have had at least some reduction of superhelical turns increased with increasing KCl concentrations (Fig. 6b and c, channels 2-6). Incomplete relaxation of nearly all PM2 DNA molecules as caused in the presence of 100-250 mM KCl (see Fig. 6c, channels 5-10) was also observed, when the reaction was performed at 0°C in the absence of KCl (Fig. 7).

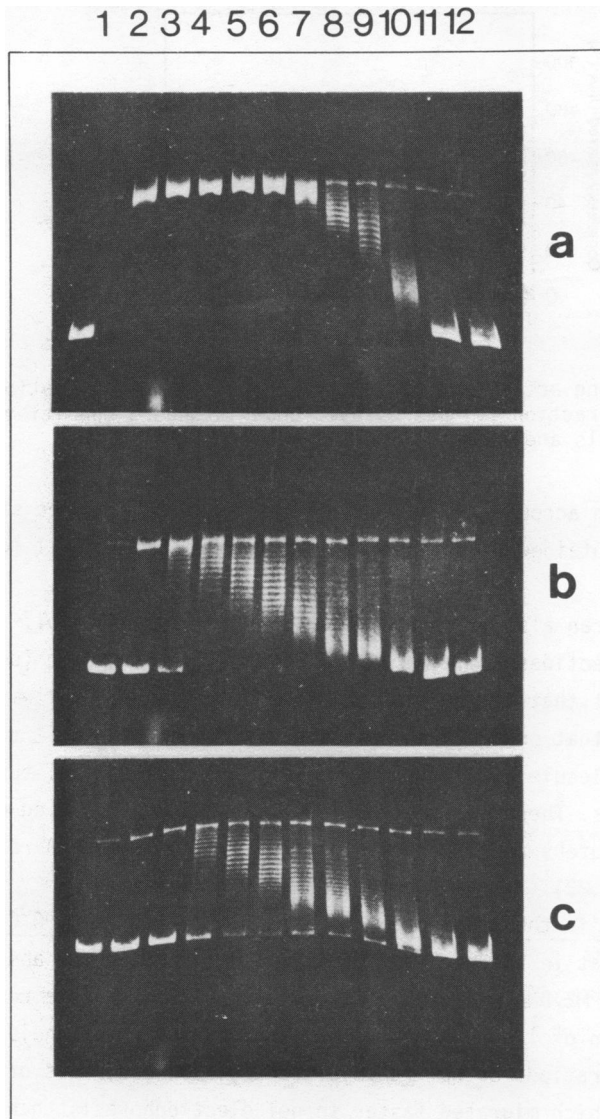


Fig. 6. DNA-relaxing activity in the presence and absence of potassium chloride. Assays were as described in Materials and Methods, except that increasing amounts of KCl were added. DNA was analysed in  $Mg^{++}$ -free gels. (a) 3.8 ng DNA-relaxing enzyme from fraction IV per assay; (b) 0.8 ng; (c) 0.4 ng. Channels: (1) and (12) controls (without enzyme); (2) no KCl; (3) 50 mM KCl; (4) 75 mM; (5) 100 mM; (6) 125 mM; (7) 150 mM; (8) 175 mM; (9) 200 mM; (10) 250 mM; (11) 375 mM.



It is possible that these results reflect the relative rates of relaxation activity and dissociation of enzyme from DNA. With low salt or at 37°C, dissociation is slow relative to unwinding activity, and therefore, there will be complete relaxation of the attacked molecules. At high salt or low temperature, dissociation is more rapid relative to unwinding activity. Thus, dissociation will occur before complete unwinding occurs, and more DNA molecules will be hit by the enzyme.

It is clear, moreover, that the determination of optimal KCl concentration for enzyme activity is not a simple task. It would require the measurement of the sum of superhelical turns removed from total DNA for each KCl concentration at a given  $Mg^{++}$  concentration.

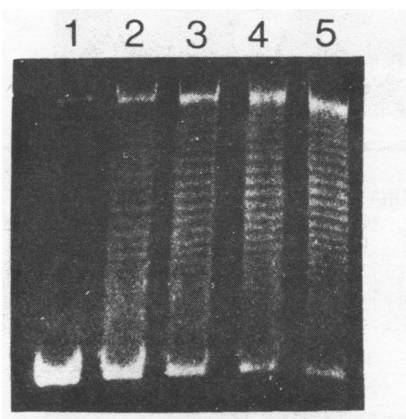


Fig. 7. Enzyme activity at 0°C. PM2 DNA was incubated with 7.5 ng DNA-relaxing enzyme for various times. DNA was analyzed in  $Mg^{++}$ -free gels. Channels: (1) control (without enzyme); (2) PM2 DNA incubated for 30 min with DNA-relaxing enzyme; (3) 60 min; (4) 90 min; (5) 120 min.

Dependence of the DNA-relaxing enzyme upon  $Mg^{++}$ . The enzyme showed no activity in the absence of  $Mg^{++}$  (Fig. 8a, channel 2). However, 0.4 mM  $MgCl_2$  in the assays sufficed to activate the enzyme (Fig. 8a, channel 3). DNA relaxation was observed using  $Mg^{++}$  concentrations between 0.4-40 mM (Fig. 8a, channels 3-13). The  $Mg^{++}$  optimum determined in the absence of monovalent cations was approximately 4-8 mM, when only complete relaxation to form  $I^0$  was taken into account (Fig. 8b). When the  $Mg^{++}$  concentration was raised above the optimum, incomplete relaxation was observed; however, more DNA molecules were attacked by the same amount of enzyme (Fig. 8b, channels 9-11).

Analysis of the limit product of the DNA relaxation process. It was reported that the DNA-relaxing enzyme of mouse cells converted closed circular DNA

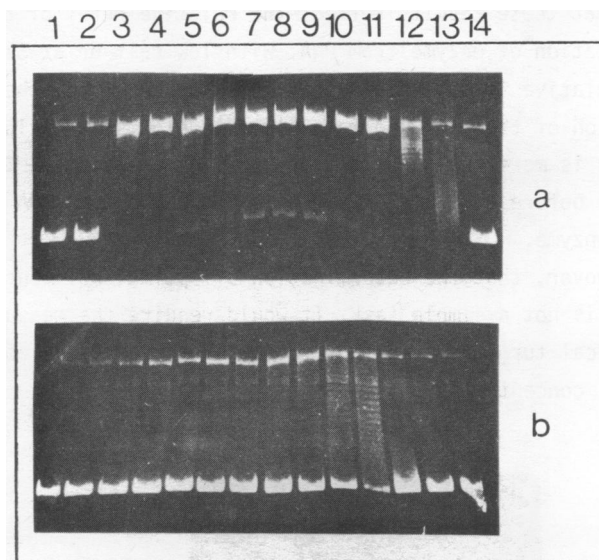


Fig. 8. Dependence of DNA relaxing activity upon  $Mg^{++}$ . In the assays (see Materials and Methods), the  $MgCl_2$  concentration was varied. DNA was analyzed in  $Mg^{++}$ -free gels. (a) 3.8 ng DNA-relaxing enzyme from fraction IV per assay; (b) 0.3 ng. Channels: (1) and (14) controls (without enzyme); (2) no  $MgCl_2$ ; (3) 0.4 mM  $MgCl_2$ ; (4) 1.4 mM; (5) 3.4 mM; (6) 4.4 mM; (7) 5.6 mM; (8) 6.9 mM; (9) 9.4 mM; (10) 14.4 mM; (11) 19.4 mM; (12) 29.4 mM; (13) 39.4 mM.

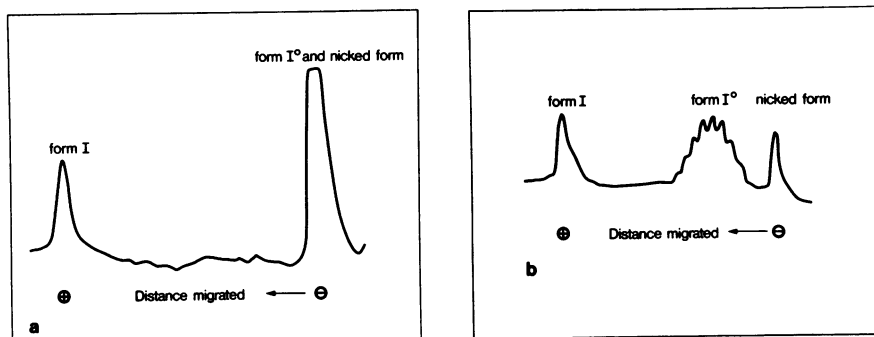


Fig. 9. Analysis of the limit product of the DNA relaxation process. In two parallel assays, PM2 DNA was incubated with 1.8 ng DNA-relaxing enzyme under standard conditions. The DNA was analysed by agarose gel electrophoresis (a) at 20°C without  $Mg^{++}$ , (b) at 4°C with 5 mM  $MgCl_2$  present in the gel and electrophoresis buffer. The gels were stained and photographed (see Materials and Methods); photographs were scanned with a Joyce-Loebl microdensitometer.

into a set of DNA species which differed in superhelical winding numbers  $\tau$  by unit values and centered around  $\tau = 0$  (15). The relative quantities of the

species followed a Gaussian distribution.

The single band in Fig. 9a suggests that relaxation of PM2 DNA by the M.luteus enzyme was complete (limit product: form I<sup>0</sup>). Conditions of electrophoresis (5 mM Mg<sup>++</sup>, 4°C (15)) which imposed higher duplex winding numbers  $\beta$  to each covalently closed circle of the limit product (generating an increase in negative superhelical turns according to  $\Delta\beta = -\Delta\tau$  (24)) resolved the single band into a set of DNA species differing in winding numbers  $\tau$ . Tracing of the band intensities by microdensitometry yielded a Gaussian-shaped curve (Fig. 9b).

## DISCUSSION

DNA-relaxing enzymes were first found in E.coli (1), subsequently in various eukaryotic cells (2,4,6,12,13) and recently in vaccinia virus (11). The DNA-relaxing enzyme described in the present paper is the second one of prokaryotic origin.<sup>1)</sup>

From the results reported here evidence suggests that the DNA-relaxing enzyme from M.luteus and the  $\omega$  protein from E.coli may be similar; both enzymes in turn differ from the eukaryotic enzymes. The following experimental data support this suggestion.

The molecular weights of both prokaryotic enzymes are very similar. Thus sodium dodecyl sulfate - polyacrylamide gel electrophoresis of the M.luteus enzyme revealed a single polypeptide chain whose mobility corresponded to 118,000  $\pm$  12,000 daltons. This molecular weight was confirmed by gel filtration. The  $\omega$  protein from E.coli was also reported to consist of a single polypeptide chain (26) having a molecular weight of 110,000 - 120,000 daltons (5,26). In contrast, the eukaryotic DNA-relaxing enzymes have molecular weights in the range of 60,000 - 70,000 daltons (6,13).

Both, the  $\omega$  protein from E.coli (1) and the DNA-relaxing enzyme from M.luteus require Mg<sup>++</sup> for activity, whereas the eukaryotic enzymes do not (2-4,6,13).

The prokaryotic  $\omega$  proteins closely resemble each other in their mode of action (5) and their dependence on superhelicity of the DNA substrate (1,5). At 37°C and low ionic strength they are capable of completely relaxing negatively twisted DNA. At 0°C or at high ionic strength, however, the enzymes generate

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<sup>1)</sup>After submission of this publication, Kung and Wang (25) also reported the purification of a DNA-relaxing enzyme from M.luteus.

intermediates indicating that the DNA-relaxing process involves many nicking and sealing events (5). In preliminary experiments we found that the M.luteus enzyme was inactive on positively twisted PM2 DNAs (generated by addition of ethidium bromide) under conditions under which the DNA-relaxing enzyme from human fibroblasts (Witte and Thielmann, unpublished) did relax these substrates. Quite in contrast to the eukaryotic enzymes (2-4,6,12), the  $\omega$  protein from E.coli is also inactive on positively twisted superhelical DNA substrates (1,5).

The kinetics of relaxation of negatively twisted superhelical DNA by the prokaryotic enzymes appears to be similar in regard to the extent of relaxation as a function of enzyme concentration as expressed in sigmoidal curves (Fig. 5, (27)). These findings suggest that both enzymes may attain their full activity as multimers. Since we did not detect multimers of the M.luteus enzyme in Sephadex G 150 column fractions (Fig. 4 b), we conclude that formation of the multimeric complex involves the DNA substrate.

One difference, however, between the  $\omega$  protein from E.coli and the DNA-relaxing enzyme from M.luteus seems to reside in their binding affinities to phosphocellulose and UV-irradiated double-stranded DNA-cellulose. As described, the M.luteus enzyme bound more tightly to phosphocellulose (elution by 0.2 - 0.4 M potassium phosphate) and to DNA-cellulose (elution by 1.8 - 2.2 M NaCl) than did the  $\omega$  protein from E.coli (0.1-0.2 potassium phosphate and 0.55-1.0 M NaCl respectively (1,26)). It is possible that the stronger binding of the M.luteus enzyme reflects the presence (or exposure) of more positively charged groups in the enzyme molecule.

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