Purification and Characterization of an \( \omega \) Protein from *Micrococcus luteus*  

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An \( \omega \) protein capable of a concerted breaking and rejoicing of DNA backbone bonds has been purified from *Micrococcus luteus* to homogeneity. The protein is a single polypeptide with a molecular weight of 120,000. It catalyzes the removal of superhelical turns from a highly negatively twisted DNA efficiently. The reaction requires \( \text{Mg}^{2+} \). In a medium containing 0.1 M \( \text{K}^{+} \), the enzyme acts in distributive fashion. It becomes more processive when the salt concentration is lowered. The rate of the reaction is strongly dependent on the sense and degree of superhelicity of the DNA substrate; with DNA substrates containing a few negative or positive superhelical turns the reaction is very slow. It appears that the prokaryotic \( \omega \) proteins isolated from *M. luteus* and *Escherichia coli* have rather similar enzymatic properties, and both differ from similar proteins from eukaryotic organisms in their \( \text{Mg}^{2+} \) requirement and in their strong dependence on superhelicity. The *M. luteus* enzyme also catalyzes the formation of knotted single-stranded DNA rings (Liu, L. F., Depew, R. E., and Wang, J. C. (1976) J. Mol. Biol. 106, 439–452). The *M. luteus* and *E. coli* \( \omega \) proteins are immunologically rather different however. Antibodies against one do not affect the activity of the other significantly.

In 1971, an *Escherichia coli* protein capable of removing superhelical turns from twisted DNAs was isolated (1). This protein, which was given an interim designation \( \omega \), was shown to require no cofactors other than inorganic salts in its action. A mechanism was proposed which interpreted the observed activities reported to date require no added \( \text{Mg}^{2+} \) and can remove both negative and positive superhelical turns efficiently. All the eukaryotic activities reported to date require no added \( \text{Mg}^{2+} \) and can remove both negative and positive superhelical turns efficiently. To test whether the unique features of the *E. coli* \( \omega \) protein are shared by similar proteins from other prokaryotic organisms, we have purified and characterized an \( \omega \) protein from the gram-positive bacterium *Micrococcus luteus*.

**EXPERIMENTAL PROCEDURES**

**Materials**

- **Bacterium**—Spray-dried *Micrococcus luteus* cells were purchased from Miles.
- **Enzymes**—*Escherichia coli* DNA ligase was a gift of Dr. P. Modrich (Biochemistry Department, Duke University Medical School). Egg white lysozyme and bovine pancreatic DNase I were purchased from Calbiochem.
- **DNAs**—Calf thymus DNA was obtained from Worthington. *Pseudomonas* phage PM2 DNA was obtained in the covalently closed form by published procedures (7). The DNA was converted to the nicked circular form by treatment with DNase I as previously described (8). Simian virus 40 (SV40) DNA was a gift from Dr. H. Katsanazsu (University of California at Los Angeles). A covalently closed SV40 DNA sample was prepared in vitro by mild treatment with DNase I, followed by treatment with *E. coli* DNA ligase in the presence of ethidium to give a superhelical density of about 0.069, taking the unwinding angle of the DNA helix caused by the binding of an ethidium molecule as 26° (10, 11). The covalently closed DNA was purified according to published procedures (8). Single-stranded phage fd DNA was a gift of Dr. L. Liu (Harvard University).
- **Antibody Preparation**—Sera of rabbits which had been injected with *M. luteus* \( \omega \) protein and *E. coli* \( \omega \) protein were provided by Animal Resources of the University of California at Berkeley. The \( \gamma \)-globulin was partially purified according to published procedures (12). The cross-reactions of antigens and antibodies were assayed by the Ouchterlony double immunodiffusion technique (13).
- **Other Materials**—Reagent grade ammonium sulfate was obtained from Mallinkrodt. Ethidium bromide was from Calbiochem. Immunodiffusion plates were purchased from Hyland Division, Travensol Laboratory. Agarose, acrylamide, and bisacrylamide were from Bio-Rad. DEAE-cellulose (type DE52) and phosphocellulose (type P11) were from Whatman. Sepharose 4B was purchased from Pharmacia. DNA-Sepharose was prepared according to the method of Kung and Wang (9). The product contained about 0.1 mg of DNA/ml of wet packed material as determined by the method of Alberts and Herrick (15).

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Methods

Protein Concentration Determination – The protein concentrations of the streptomycin sulfate fraction, the ammonium sulfate fraction, and the DEAE-cellulose fraction were determined by the microbiuret method (16). The protein concentrations of the phosphocellulose fraction and purified ω protein were determined according to Lowry et al. (17). We also measured the concentrations of a purified M. luteus ω sample by both microbiuret and Lowry methods, using bovine plasma albumin as the standard. Both methods gave the same value. Based on this measured concentration the absorbance at 280 nm of a 1% M. luteus ω solution was determined to be 0.2.

Fluorescence Assay – The fluorescence assay method described by Morgan and Pulleyblank (18) was used to measure the activity of ω protein during purification. The assay mixture (200 µl) was 70 mM potassium phosphate (pH 8), 4 mM MgCl₂, 1 mM EDTA containing 10 µg/ml of native F2M2 DNA, and 50 µg/ml of bovine serum albumin. One unit of activity of ω protein is arbitrarily defined as the amount required to give one-half of the maximal fluorescence drop in 30 min at 37°C. Per assaying the streptomycin sulfate and ammonium sulfate fractions, 3 µg of E. coli tRNA, which does not inhibit ω activity, was added to each assay mixture to inhibit an endonuclease contaminant. After the DEAE-cellulose step, the ω protein and the endonucleolytic activity were separated, and tRNA was not added for the assays.

Gel Electrophoresis – Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of protein was done according to the published procedures (19, 20).

Electrophoresis of DNA was performed in slab gels of agarose. After electrophoresis, the gels were stained and photographed. The negatives were traced with a Joyce-Loebl microdensitometer. All the procedures had been previously described (21).

Analytical Ultracentrifugation – Band and boundary sedimentation measurements were performed in a model E analytical ultracentrifuge (Spinco) as previously described (22, 23).

RESULTS

Purification of Micrococcus luteus ω Protein

Unless otherwise stated, all operations were carried out at 4°C.

Lysis of Cells – Three hundred grams of spray-dried M. luteus cells were washed in 2 liters of 10 mM Tris-HCl (pH 7.8). The cells were collected by centrifugation at 10 krpm for 10 min, then resuspended in 6 liters of 10 mM Tris-HCl (pH 7.8), 0.2 M sucrose. The solution was warmed to 37°C, and 900 mg of lysozyme dissolved in 100 ml of 10 mM Tris-HCl (pH 7.8) was added with stirring. After 15 min, 0.3 ml of 1 M MgCl₂ was added. Lysis was complete after another 25 min; the absorbance at 650 nm dropped to 4% of its original value. The lysate was cooled to 4°C.

Streptomycin Sulfate Fractionation – The viscous lysate was diluted with 6 liters of 0.1 M Tris-HCl (pH 7.6) and 60 ml of 1 M MgCl₂, then stirred manually to give a uniform solution. A total of 960 ml of 10% (w/v) freshly prepared streptomycin sulfate was added dropwise to the lysate with stirring. After all the streptomycin sulfate was added, the suspension was stirred for another 30 min, and then centrifuged at 12 krpm for 40 min. The supernatant was collected (Fraction I).

Ammonium Sulfate Fractionation – Solid (NH₄)₂SO₄ (6.28 kg) was added to Fraction I (13.4 liters) over a period of 2 h with stirring. The suspension was stirred for another hour, and then centrifuged at 12 krpm for 1 h. The precipitate was collected and then extracted with 2 liters of 50% saturated (NH₄)₂SO₄ solution (in 50 mM Tris-HCl, pH 7.6). After stirring for 1 h, the precipitate was collected by centrifugation and extracted again with 2 liters of 25% saturated (NH₄)₂SO₄ solution. The pellet was removed by centrifugation and the volume of the supernatant (Fraction II) was 2.0 liters.

DEAE-cellulose Chromatography – Fraction II was dialyzed against three changes of 10 mM Tris-HCl (pH 7.6), 0.1 M NaCl (20 liters each and 6 h per change), and then applied at a flow rate of 500 ml/h to a DEAE-cellulose column (58 cm x 47 cm²) equilibrated with 10 mM Tris-HCl (pH 7.6), 0.1 M NaCl. The column was washed with 4 liters of the same buffer, and then eluted with a 20-liter linear gradient of NaCl (0.1 to 0.4 M) containing 10 mM Tris-HCl (pH 7.6). Fractions containing ω activity, which was eluted between 0.14 M and 0.2 M, were pooled (Fraction III, 4.1 liters).

Phosphocellulose Chromatography – Fraction III was applied at a flow rate of 230 ml/h to a phosphocellulose column (28 cm x 31 cm²) which had been equilibrated with 10 mM Tris-HCl (pH 7.6), 0.17 M NaCl. The column was washed with 900 ml of the same buffer, and then eluted with an 8-liter linear gradient of NaCl (0.17 to 1 M) containing 10 mM Tris-HCl (pH 7.6). The ω activity was eluted between 0.2 M and 0.32 M of NaCl. These fractions were pooled (Fraction IV, 1.1 liters).

DNA-Sepharose Chromatography – Fraction IV, after the addition of 60 ml of glycerol, was applied at a flow rate of 40 ml/h to a DNA-Sepharose column (30 cm x 1.66 cm²), which had been equilibrated with 10 mM Tris-HCl (pH 7.6), 0.25 M NaCl, 5% glycerol. The column was first washed with 90 ml of the equilibrating buffer, then eluted stepwise with 10 mM Tris-HCl (pH 7.6), 5% glycerol, 0.5 mM EDTA, and quantities of NaCl as described below. The eluant for the first two steps (90 ml each) contained 0.4 M and 0.8 M NaCl, respectively. The eluant for the third step contained 4.2 M NaCl and 0.1 M MgCl₂. The flow rate for the last step was 16 ml/h. The ω activity was found in the first 20 ml of the 4.2 M NaCl, 0.1 M MgCl₂ eluant. A summary of the purification steps is given in Table I.

PROPERTIES OF M. LUTEUS ω PROTEIN – Electrophoresis of the purified protein in sodium dodecyl sulfate-polyacrylamide gel reveals a single band. The mobility of the band corresponds to a molecular weight of 120,000, which is slightly higher than that of the Escherichia coli ω protein (110,000). In 0.2 M KCl, 0.02 M potassium phosphate, pH 6.5, and at 20°C and a protein concentration of 160 µg/ml, M. luteus ω sediments with a sedimentation coefficient of 5.0 S. From preliminary analysis of the amino acid composition of the protein we estimated that its partial specific volume is about 0.719 cm³ g⁻¹ (24). Assuming that the protein is monomeric, its frictional coefficient is calculated from the sedimentation coefficient to be 1.79 times that of an unhydrated sphere of the same molecular weight. If the protein is dimeric, the corresponding frictional coefficients ratio is 2.23 x 1.79 or 2.0. We feel that the lower value is more reasonable, and therefore, the protein probably exists in solution as monomers each containing a single polypeptide chain of molecular weight 120,000.

The protein has a broad pH optimum centering around pH 8.

TABLE I

<table>
<thead>
<tr>
<th>Purification of ω protein from 300 g of spray-dried Micrococcus luteus cells</th>
<th>Step</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Yield *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total protein</td>
<td>Total activity</td>
<td>Specific activity</td>
<td>Yield *</td>
<td></td>
</tr>
<tr>
<td></td>
<td>µg/ml</td>
<td>units</td>
<td>µg/ml</td>
<td>units/mg</td>
<td>%</td>
</tr>
<tr>
<td>Streptomycin sulfate</td>
<td>40 g</td>
<td>20</td>
<td>5.0</td>
<td>10²</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>16.6 g</td>
<td>13</td>
<td>7.8</td>
<td>10²</td>
<td>65</td>
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<tr>
<td>DEAE-cellulose</td>
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<td>10</td>
<td>1.8</td>
<td>10²</td>
<td>50</td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>231 mg</td>
<td>2.9</td>
<td>1.2</td>
<td>10²</td>
<td>15</td>
</tr>
<tr>
<td>DNA-Sepharose</td>
<td>5 mg</td>
<td>2.6</td>
<td>5.0</td>
<td>10⁶</td>
<td>39</td>
</tr>
</tbody>
</table>

* Yield normalized by taking the streptomycin sulfate fraction as 100%.
and the optimal temperature is about 37°C. The removal of DNA superhelical turns by *M. luteus* ω requires Mg2+. When assayed in the standard assay medium containing 70 mM potassium phosphate, 4 mM MgCl2, 1 mM EDTA, pH 8.1, the addition of spermidine to 1 to 3 mM has no stimulating effect. The reaction is not inhibited by 1 to 10 mM N-ethylmaleimide. The *M. luteus* protein binds strongly to single-stranded DNA. Removal of the protein from single-stranded DNA-Sepharose column requires a nearly saturated NaCl solution. Because of this strong binding, the relaxation of a superhelical DNA substrate is strongly inhibited by single-stranded DNA. These aspects are similar to the *E. coli* ω protein.

No cross-reaction is detectable when the Ouchterlony double immunodiffusion assays are done with *E. coli* and *M. luteus* ω proteins and antibodies against the individual proteins. Nor does the addition of antibodies against one affect the activity of the other in standard assays. Thus immunologically the two ω proteins are rather distinct.

**Role of ω Protein Is Catalytic** – The catalytic nature of *M. luteus* ω protein is demonstrated by the experiment illustrated in Fig. 1. Superhelical SV40 DNA was treated with different amounts of ω protein and the products were examined by gel electrophoresis. Fig. 1a depicts the electrophoretic pattern of the DNA before treatment with ω. After 0.1 µg of the DNA was incubated with 0.15 ng of the protein, in 50 µl of a medium containing 70 mM potassium phosphate, 4 mM MgCl2, 1 mM EDTA, pH 8.1, its electrophoretic pattern changed to the one shown in Fig. 1b. There is no overlapping between the distributions of the covalently closed DNA bands of the samples before and after reaction, clearly showing that some superhelical turns have been removed from each and every DNA molecule. Taking the molecular weights of SV40 DNA and *M. luteus* ω protein as 3.4 × 106 and 120,000, respectively, it immediately follows that about 30 DNA molecules are relaxed per protein molecule. Thus the protein is an enzyme capable of removing superhelical turns catalytically. With this limited amount of ω protein, not all the superhelical turns have been removed. As illustrated in Fig. 1c, further reduction in the number of negative superhelical turns is evident if the enzyme concentration is tripled. The results shown in Fig. 1 also indicate that the action of ω in the medium used is nonprocessive. All DNA molecules are affected in the presence of a much smaller number of protein molecules. If the potassium phosphate concentration is lowered to below 30 mM, the reaction pattern is changed. If there are fewer protein molecules than DNA molecules, the degree of superhelicity of a fraction of the DNA remains unchanged.

**Dependence of Reaction on Superhelicity of DNA Substrate** – In the presence of a large amount of the *M. luteus* ω protein, the removal of superhelical turns from a negatively twisted DNA is complete. This is demonstrated by an experiment shown in Fig. 2. Fig. 2a depicts the electrophoretic pattern of a PM2 DNA sample, which had been first treated with bovine pancreatic DNase I to introduce a few single chain scissions into each molecule and then treated with ligase. This cycle of nicking and joining results in a population of covalently closed DNA molecules with topological winding numbers representative of such molecules at thermal equilibrium at the temperature and in the medium the ligase reaction was carried out (21). The electrophoresis conditions were chosen such that the covalently closed DNA molecules became twisted so that species of different topological winding numbers would resolve. Fig. 2b depicts the electrophoretic pattern of a PM2 DNA sample, originally negatively twisted, after treatment with *M. luteus* ω at a temperature and in a medium identical with the ligase reaction just described. The patterns for the covalently closed DNA species shown in Fig. 2a and b, are virtually identical, showing that the removal of the superhelical turns by *M. luteus* ω is complete.

We have also tested the removal of turns by the *M. luteus* enzyme from a positively twisted DNA. Fig. 2, c and d, illustrate such an experiment. PM2 DNA containing a few positive turns was prepared by ligase closure of nicked PM2 DNA at a temperature lower than the temperature at which the reaction with ω was carried out (26). Its electrophoretic pattern is shown in Fig. 2c. After prolonged incubation of the DNA with a high concentration of the *M. luteus* enzyme, the positive turns are removed, as shown by the pattern depicted in Fig. 2d, which is the same as that of a sample containing no superhelical turns (Fig. 2a) at the temperature and in the medium the reaction with ω was carried out. We note that for all the samples shown in Fig. 2, during electrophoresis, the covalently closed DNA species are negatively twisted. A closed circular DNA with a lower topological winding number is more negatively twisted and therefore migrates faster than the same DNA with a higher topological winding number. Removal of positive turns by ω means a reduction in the...
One of us has reported previously that the E. coli \( \omega \) protein catalyzes the formation of knotted DNA rings \((22)\). The knotted form is characterized by a higher sedimentation coefficient in an alkaline medium than that of the normal circular form. We have found that the M. luteus enzyme can carry out the same reaction. In a typical experiment, single-stranded coliphage fd DNA and M. luteus \( \omega \) protein were incubated in a medium containing 50 mM Tris·HCl (pH 8), 160 mM KCl, 3 mM MgCl\(_2\), and 50 \( \mu \)g/ml of bovine plasma albumin. The final concentrations of the DNA and \( \omega \) protein were 18 \( \mu \)g/ml and 6 \( \mu \)g/ml, respectively. After 30 min at \( 37^\circ \), the reaction was terminated by the addition of EDTA to 0.02 M. Sedimentation of the sample in an alkaline medium containing 3 M CsCl, 0.04 M KOH, 0.01 M EDTA revealed that approximately 30% of the input circular DNA had been converted to a species which sediments 1.2 times faster. This reaction is completely analogous to the formation of knotted DNA rings by E. coli \( \omega \) protein \((22)\). The reaction requires \( \text{Mg}^{2+} \) and is blocked by antibodies against M. luteus \( \omega \). Also, similar to the reaction with E. coli \( \omega \), linear monomer-sized and fragments of fd DNA are also formed in the reaction described above. These linear species are formed from an alkali-labile DNA·\( \omega \) complex. Exposure of such a complex to alkaline leads to the breaking of a DNA backbone bond and the linking of the \( \omega \) protein to the \( 5' \)-end of the polynucleotide. Detailed results will be presented elsewhere.

**DISCUSSION**

Although the *Escherichia coli* and *Micrococcus luteus* \( \omega \) proteins are immunologically rather different, their enzymatic characteristics are very similar. Both act efficiently on highly negatively twisted (underwound) DNA substrates, but relax covalently closed DNAs containing a few negative or positive superhelical turns only at high protein concentrations and after prolonged incubation. With single-stranded circular DNA as substrate, both \( \omega \) proteins catalyze the formation of knotted rings. Both proteins require \( \text{Mg}^{2+} \) but not other cofactors for these reactions. The dependence on \( \text{Mg}^{2+} \) and the degree and sense of superhelicity of the DNA substrate sets these prokaryotic proteins apart from similar activities found in eukaryotic organisms \((2-6)\).

Since there is only scant information on the biological functions of \( \omega \) type activities, discussion on the possible significance of the difference in superhelicality requirements between the prokaryotic and the eukaryotic activities is naturally speculative. The difference might be due to differences in the functions of the eukaryotic and prokaryotic \( \omega \) proteins, or to the loss of factors during the purification of the prokaryotic enzymes which are needed for the efficient relaxation of positively twisted DNA. Cellular functions capable of promoting the unwinding of the DNA duplex might also eliminate the requirement of negative superhelicity.

Another possibility might lie in a basic difference between
the supercoiling of eukaryotic and prokaryotic DNAs inside the cells. It appears that both prokaryotic and eukaryotic DNA are organized in vivo in such a way that the DNA is underwound relative to the winding number expected on a B form helix (27-33). In an eukaryotic organism, the underwinding of the DNA appears to be at least in part due to the formation and organization of the nucleosomes (32, 33). The condensation and decondensation of DNA, in addition to other cellular processes, are expected to require activities which can remove both negative and positive superhelical turns (3). In E. coli, recent results indicate that the underwinding of the DNA is caused by an ATP-requiring activity termed gyrase (34, 35). This enzyme can reduce the topological winding number of a covalently closed DNA until the DNA is highly negatively twisted (34). Similar to an ω protein, the gyrase must also possess or contain an activity which can break and rejoin the DNA backbone. If prokaryotic cellular DNA is always driven to the underwound state by gyrase, then negatively twisted DNA is the natural substrate of prokaryotic ω proteins.

Mechanistically, the strong dependence of the ω-catalyzed relaxation of a DNA supercoil on the degree and sense of superhelicity can be interpreted as due to the formation of an obligatory ω-DNA complex in which the DNA helix is unwound somewhat (1). The formation of this complex precedes the formation of the putative intermediate in which a DNA backbone bond is broken and a DNA-protein bond is formed (1). For negatively twisted DNA, the formation of a complex in which the DNA helix is locally unwound is associated with a reduction in the number of negative superhelical turns, and is therefore thermodynamically more favorable comparing with the same reaction with an untwisted or a positively twisted DNA (36, 37). The dependence of the ω reaction on superhelicity can be compared with the superhelicity dependence of RNA synthesis by E. coli RNA polymerase. The core enzyme of E. coli RNA polymerase is incapable of unwinding the DNA helix (38), and is virtually inactive with a covalently closed but untwisted circular DNA as the template (8). With a negatively twisted DNA template, the core enzyme is rather active (8). Similarly, transcription by the E. coli RNA polymerase holoenzyme is also strongly affected by the degree of superhelicity (39-41).

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REFERENCES