Two-dimensional crystallization of DNA gyrase B subunit on specifically designed lipid monolayers

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The B subunit of DNA gyrase formed two-dimensional crystals when bound to a specifically recognized phospholipid spread into a monolayer at the air/water interface. The especially designed lipids consisted of novobiocin coupled through the 3′ or 2″ hydroxyl group and a hydrophilous linker of a given length to dioleoylphosphatidic acid. Two-dimensional crystals of the gyrase B subunit are formed under physiological conditions of pH and ionic strength, with no precipitant added to the solution. Crystal diffraction extended to a 2.7 nm resolution in negative stain, with unit cell parameters $a = 6.1$ nm, $b = 7.6$ nm and $\gamma = 64°$.

DNA gyrase, Novobiocin, Protein-lipid interaction, Two-dimensional crystallization; Electron microscopy; Image processing

1. INTRODUCTION

DNA topoisomerases are enzymes that control and modify the topological state of DNA and play a crucial role in determining the function of DNA in cells. These enzymes catalyze many conversions between different topoisomers of DNA [1–4]. DNA gyrase is a prokaryotic type II topoisomerase and is distinct from other topoisomerases in being able to convert relaxed circular DNA to a superhelical form. The energy needed for the conversion is supplied by ATP hydrolysis. The enzyme was first isolated from *E. coli* [5] and has also been detected in other organisms [6,7]. It catalyses a number of reactions such as negative supercoiling of close circular duplex DNA (ATP dependent), relaxation of superhelical DNA (in absence of ATP), introduction of double strand breaks at specific sites in DNA (promoted by oxolinic acid), hydrolysis of ATP (DNA dependent) and formation and resolution of catenated and knotted duplex DNA.

DNA gyrase is an A_2B_2 tetramer [8,9]. Subunit A has a molecular mass of 105000 Da and is encoded by the nal A gene while subunit B, whose molecular mass is 95000 Da, is produced by the cou gene [10]. DNA gyrase is inhibited by two classes of antibiotics. The first one, including nalidixic and oxolinic acids and analogs, acts directly on the gyrase A subunit that mediates the ability of the enzyme to introduce and rejoin double strand breaks in DNA [11–13]. The target of the second class of antibiotics, of which are coumermycin A1 and novobiocin, is the gyrase B subunit. These drugs interact competitively with ATP and prevent energy transduction [10,14–16]. Both A and B subunits are required to reconstitute the full enzyme activity [8].

Recently, direct observation by electron microscopy of the gyrase A subunit dimer, of the B subunit monomer, of the holoenzyme and of its complex with DNA were reported [17]. Authors showed that the unique shapes of the A_2B_2 tetramer and of the A_2 dimer make it possible to deduce the relative locations of the subunits in the holoenzyme and to indicate a plausible path of the DNA segment in the gyrase DNA complex. Images of these complexes were obtained by spraying the enzyme onto mica and further contrast enhancement visualized by rotary shadowing with platinum. A three-dimensional computer simulation of gyrase was obtained by direct-space reconstruction of the Pt shadowed outline of the molecule. The effective resolution was limited to about 30 Å and was affected by Pt grain size and distortions of adsorbed molecules to mica.

This report concerns the preliminary results obtained in two-dimensional crystallization of the gyrase B subunit at the interface between a solution of the protein and monolayers containing novobiocin derivatized phospholipids, specifically recognized by this protein. The two-dimensional crystallization technique was introduced in 1983 [18] and since that, appears to be more and more powerful, due to the developments in

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the design of the lipid structures, in electron microscopy techniques and image processing.

2. MATERIALS AND METHODS

Different novobiocin derivatized phospholipids were designed and synthesized for this study (Scheme 1), accounting for considerations of monolayers stability and fluidity, affinity of the gyrase B subunit for the novobiocin derivatives and accessibility of the modified drug for the protein [19].

Stability and fluidity of phospholipid monolayers are essentially governed by the nature of the acyl chains on the glycerol backbone. According to previous results [20], dioleoyl chains appeared to provide the monolayers made of these lipids with enough stability and fluidity. Such monolayers are in a fluid phase between 4 and 37°C, which is convenient when working with biological material.

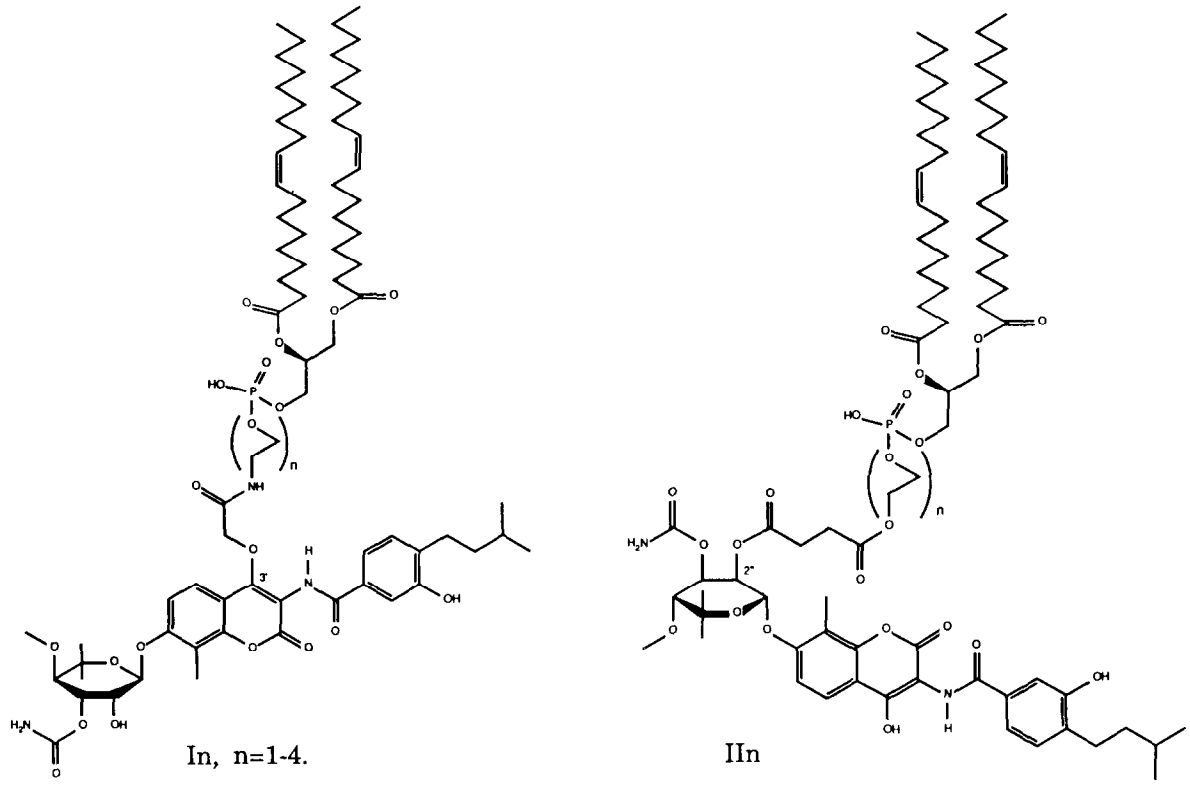
The way novobiocin is attached to the lipid is decisive for the affinity of the gyrase B subunit towards the ligand. Fragmentary results of the literature concerning the affinity of the protein for novobiocin derivatives [21] led us to link the antibiotic to the lipid in two different manners (through the 3′ and 2′′ positions, Fig. 1). However high the affinity of the protein for the modified drug bound to the phospholipid can be, the accessibility of the ligands seems to remain a crucial point in obtaining high quality protein arrays. That accessibility is directly dependent on the length of the spacer between the lipid and the drug and is increased from compounds I_1 and II_1 to I_4 and II_4.

The gyrase B subunit was purified from the overexpressing strain N4830/pMK461 [22], following essentially the procedures previously described [16,23]. A P_{11} phosphocellulose chromatography [24] was inserted after the Polymin P fractionation. The purity of the subunit was assayed by SDS gel electrophoresis and supercoiling activity in the presence of 5 ng of pure gyrase A subunit [22]. The gyrase B subunit was stable in our experimental conditions for more than two days as it was controlled through the DNA supercoiling activity of the reconstructed A_2B_2 tetramer.

Typically, a solution of the protein of interest (17 μl, 100 to 250 μg/ml) in buffer A (5% glycerol, 50 mM Tris pH 7.5, 1 mM EDTA, 5 mM DTT) was placed in a small teflon well (4 mm diameter, 1 mm deep). The novobiocin linked phospholipid (0.5 to 0.75 μl, 0.5 mg/ml in chloroform/hexane/methanol, 5:6:1 by vol.) was spread on the surface of the aqueous solution and the whole device was sealed and stored in the dark to prevent water evaporation. The protein arrays were allowed to form over periods ranging from a few minutes to several days at room temperature. The monolayers containing presumably arrays of protein were picked up on carbon coated electron microscope grids, negatively stained with uranyl formate and examined in a Philips 300 electron microscope. Micrographs were taken at a magnification of 45000 ×, using 60 kV electrons.

The original micrographs were digitized on the microdensitometer developed by the GSTS (Groupement Scientifique de Télédétection Spatiale) and the SERTIT (Service Régional de Traitement d'Image et de Télédétection) at 25 μm raster size producing a pixel spacing of 0.55 nm. The image processing was performed using the IMAGIC software kindly provided by M. van Heel [25], which was adapted to the SUN-UNIX environment.

Crystal images were stored as 1024 × 1024 points and the most coherent 256 × 256 areas were selected and multiplied by a smooth edged circular mask of 128 pixels in diameter. Fourier transforms were calculated for these masked areas and displayed as power spectra. Strong peaks were interactively selected and used to calculate the reciprocal lattice. Amplitude and phase of the peaks having amplitudes at least 1.5 times above background and falling on the reciprocal lattice were selected from the Fourier transform. A filtered image was then calculated by back-Fourier transforming the masked-off peaks. The filtered image was centered on a putative two-fold symmetry axis by aligning translationally the filtered image against with its 180° rotational transform.

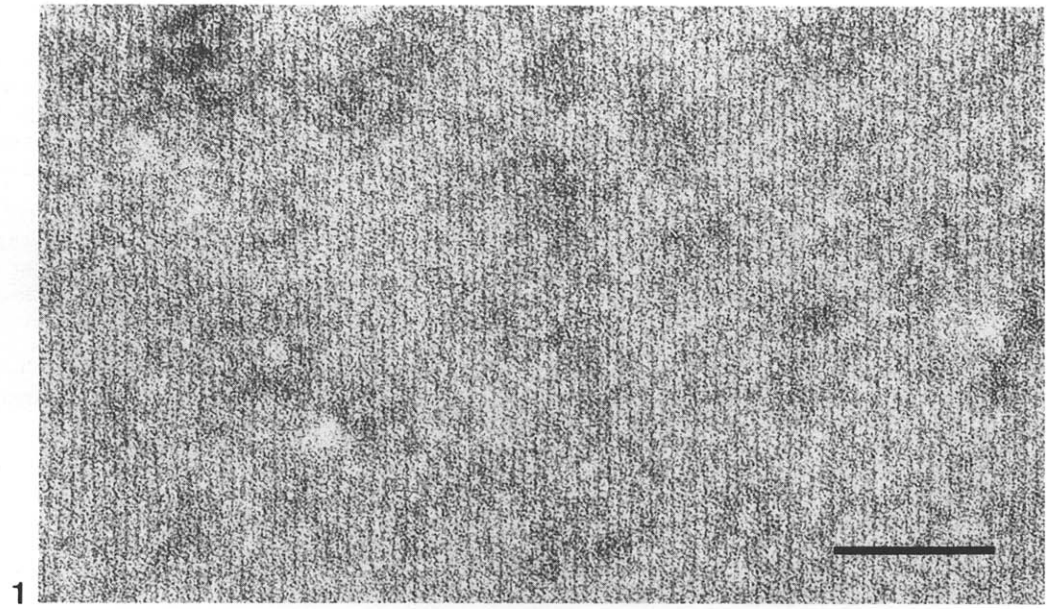


Scheme 1. Novobiocin linked phospholipids used to get two-dimensional crystals of the gyrase B subunit.

3. RESULTS AND DISCUSSION

Two-dimensional crystals of gyrase B subunit bound to a lipid monolayer were formed after a minimum incubation time of 5 h. The size and coherence of crystalline areas gradually grew over the period of incubation. However, over 48 h the quality of the crystals could not be improved any more. Crystals were obtained using protein purified according to different procedures, with various purity grades (30–90%) and at different concentrations (50–250 μg/ml).

When egg phosphatidyl choline was spread onto a monolayer onto a buffer A solution containing the gyrase B subunit, some protein was adsorbed on the lipid film but it never gave any ordered array of macromolecules. The adsorption was completely avoided by increasing the ionic strength of the solution (KCl 500 mM). Using novobiocin linked phospholipids, proteins still bound to the film with KCl concentrations up to 2 M. However, no two-dimensional crystal of the gyrase B subunit was obtained over KCl 300 mM. With poorly purified protein (30% pure), crystals were obtained using protein concentrations up to 200 μg/ml whereas highly pure protein (90% pure) gave ordered arrays even at 50 μg/ml. From these observations, we can conclude that the gyrase B subunit specifically



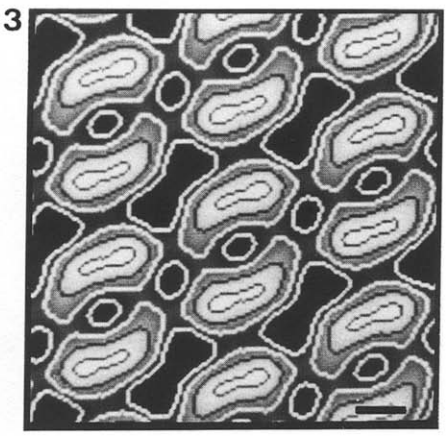


Fig. 1. Partial view of a micrograph showing a negatively stained two-dimensional crystal of the gyrase B subunit. This array was obtained using a protein concentration of 250 μg/ml in 15% glycerol, 150 mM KCl, 50 mM Tris (pH 7.5), 5 mM DTT and 1 mM EDTA, after 24 h incubation at 20°C. Bar = 100 nm.

Fig. 2. Optical diffractogram of a gyrase B subunit two-dimensional crystal. The unit cell parameters are: a = 6.1 nm, b = 7.6 nm and γ = 64°.

Fig. 3. Two-dimensional symmetrized and contoured projection map of the gyrase B subunit, obtained through a Fourier synthesis using 12 terms up to 1/2.7 nm^{-1}. Bar = 2 nm.

binds to novobiocin linked phospholipids and subsequently is oriented in a plane.

Crystallization experiments with novobiocin linked phospholipids and buffer A containing free novobiocin (0.3 mg/ml) competing with the derivatized lipids did not provide protein arrays any more, providing additional evidence for the specific molecular recognition of the ligand by the protein on the lipid monolayer.

All novobiocin linked phospholipids (I_1–I_4 and II_1–II_4) were experimented (about ten times each) and compared with regard to the quality of the protein arrays produced. Whatever the way novobiocin was linked to the lipid (through the 3′ or 2′′ position), two-dimensional crystals were obtained, showing that in all cases the affinity of the protein for the modified drug was high enough. With derivative I_3 however, the crystals obtained were definitely larger and contained less defaults than with other lipids. This observation brought to the fore the role of the length of the spacer between the lipid and the ligand specifically recognized by the protein. A shorter spacer (I_1, I_2) would likely force the protein to penetrate the lipid monolayer to complex the drug, and subsequently introduce some disorder in the array. On the other hand, a too long spacer (I_4) could provide the bound protein with too much freedom and increases the possibility to have defaults in the crystal.

Experiments with novobiocin linked phospholipids mixed with egg phosphatidyl ethanolamine, egg phosphatidyl choline, dioleoyl phosphatidyl ethanolamine or dioleoyl phosphatidyl choline in various ratios did not provide any two-dimensional arrays of the gyrase B subunit. These results are rather unexpected, due to the specific area of the lipids used that is far lower (about a hundred times) than that of the cross-section of the protein. Actually, we cannot propose any clear explanation for this phenomenon. It could be due to a modification of the environment (topology, local charge...) at the surface of the lipid film. This hypothesis assumes much importance for the two-dimensional crystallization technique of proteins and is actually under investigation.

Addition to the protein solution of a nonhydrolyzable ATP derivative, APPNP (0.3 mg/ml), improved the quality of the crystalline arrays obtained. The presence of spermidine (3 mM) provided comparable results.

Fig. 1 shows an untilted electron image of a negatively stained two-dimensional crystal of gyrase B subunit. This patch was about $1.1 \times 0.7\ \mu m$ in extent and was crystalline over virtually its whole surface.

The optical diffractogram of this ordered array is shown in Fig. 2. All sufficiently ordered arrays made from the gyrase B subunit showed the same optical diffraction pattern. The parameters of the unit cell measured from these patterns are $a = 6.1$ nm, $b = 7.6$ nm and $\gamma = 64°$. Diffraction to at least 2.7 nm was clearly observed. The phase origin of the diffraction peaks was refined to identify a possible two-fold symmetry axis, perpendicular to the crystal plane. The phases were then close to 0 or 180°, consistent with p_2 symmetry. Fig. 3 shows a two-dimensional symmetrized and contoured projection map, synthesized using 12 reflections up to $1/2.7\ nm^{-1}$, with a mean phase residual of 7.6°. The protein looks like a bean with two asymmetrical lobes. The unit cell contains two gyrase B subunits, which is reminiscent of the A_2B_2 structure of the full enzyme. The subunits are laid head to tail and connected together at two different points.

The method of forming two-dimensional crystals of proteins has several advantages. It needs very low quantities of protein (a few micrograms are sufficient) and due to the highly specific binding of the protein to the derivatized lipids, it is quite possible to work with partially purified proteins. These points are especially important when working with a protein that is very scarce or difficult to purify.

We showed by using novobiocin derivatized phospholipids especially designed for two-dimensional crystallization of DNA gyrase experiments, that the gyrase B subunit was specifically bound to the lipid monolayer and unequivocally oriented in the plane to form two-dimensional arrays.

Attention is now directed towards getting tilted images of the crystalline arrays and reconstructing the three-dimensional structure of the gyrase B subunit. In the same way, we are involved in two-dimensional crystallization of the reconstructed A_2B_2 DNA gyrase.

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