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A 4.2 kDa Synthetic Peptide as a Potential Probe to Evaluate the Antibacterial Activity of Coumarin Drugs

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Abstract: The coumarin antibiotics are potent inhibitors of DNA replication whose target is the enzyme DNA gyrase, an ATP-dependent bacterial type II topoisomerase. The coumarin drugs inhibit gyrase action by competitive binding to the ATP-binding site of DNA gyrase B protein. The production of new biologically active products has stimulated additional studies on coumarin–gyrase interactions. In this regard, a 4.2 kDa peptide mimic of DNA gyrase B protein from *Escherichia coli* has been designed and synthesized. The peptide sequence includes the natural fragment 131–146 (coumarin resistance-determining region) and a segment containing the gyrase–DNA interaction region (positions 753–770). The peptide mimic binds to novobiocin ($K_a = 1.4 \pm 0.3 \times 10^5$ M^{-1}), plasmid ($K_a = 1.6 \pm 0.5 \times 10^6$ M^{-1}) and ATP ($K_a = 1.9 \pm 0.4 \times 10^3$ M^{-1}), results previously found with the intact B protein. On the other hand, the binding to novobiocin was reduced when a mutation of Arg-136 to Leu-136 was introduced, a change previously found in the DNA gyrase B protein from several coumarin-resistant clinical isolates of *Escherichia coli*. In contrast, the binding to plasmid and to ATP was not altered. These results suggest that synthetic peptides designed in a similar way to that described here could be used as mimics of DNA gyrase in studies which seek a better understanding of the ATP, as well as coumarin, binding to the gyrase and also the mechanism of action of this class of antibacterial drugs. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptides; affinity chromatography; fluorescence; peptide synthesis; solid phase; DNA gyrase; coumarins

INTRODUCTION

DNA gyrase is a bacterial type II topoisomerase that is responsible for maintaining the topological state of DNA. Gyrase catalyses the energetically unfavourable negative supercoiling of DNA by coupling this reaction to the hydrolysis of ATP [1,2]. The enzyme from *Escherichia coli* consists of two proteins, A (GyrA) and B (GyrB), of molecular masses 97 and 90 kDa, respectively, which form an A_2B_2 active complex. In addition to DNA supercoiling, gyrase can also catalyse the ATP-independent relaxation of supercoiled DNA. Mechanistic studies have revealed the steps involved in the supercoiling reaction. Briefly, this process involves the wrapping of a segment of DNA around the enzyme, the cleavage of the wrapped DNA in both strands with the formation of covalent bonds between the newly formed 5′ phosphates and Tyr-122 of GyrA, the passage of another segment of DNA through this

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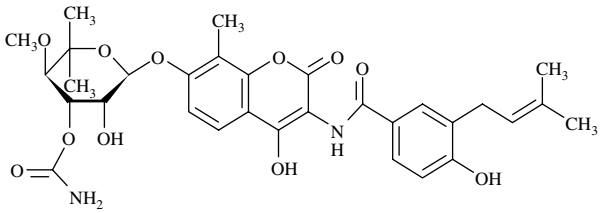
double-strand break, and resealing of the broken DNA. The result is the introduction of two negative supercoils whose driving force comes from the hydrolysis of two molecules of ATP [1–3].

A number of researchers have demonstrated that the GyrA and GyrB proteins contain distinct domains. GyrA is functionally divided into a 64 kDa *N*-terminal and a 33 kDa *C*-terminal domain, mainly involved in DNA breakage-reunion and DNA wrapping, respectively [4,5]. The B protein comprises an *N*-terminal domain (43 kDa) containing the ATP-binding site [6] and a 47 kDa *C*-terminal domain that interacts with GyrA and DNA [7]. The *N*-terminal domain includes two subdomains (24 kDa *N*-terminal part and 19 kDa *C*-terminal part). The ATP binding site is located in the first subdomain [8].

Gyrase is a target of several classes of antibacterial agents (for a review, see reference [9]), the best studied being the quinolones (e.g. norfloxacin) [10] and coumarins (e.g. novobiocin) [11] drugs. Quinolones are believed to interfere with the catalytic cycle of gyrase by interactions with subunit A of the enzyme [12], while coumarins interact with the subunit B [13]. The coumarin antibiotics are natural compounds inhibiting the gyrase action by competitively binding to the ATP-binding site in the 24 kDa subdomain of GyrB protein [3,11], as previously shown by the crystal structures of the 24 kDa gyrase subdomain–inhibitor complexes [13,14].

The coumarins are potent inhibitors of the gyrase supercoiling and ATPase reactions, however, they have failed to become clinically successful due to poor cell penetration, low solubility and toxicity in eukaryotes [9]. Despite this, the fact that these compounds are significantly more potent in inhibiting DNA gyrase *in vitro* than the quinolones has stimulated interest with regard to improving their properties in order to produce structurally related compounds suitable for clinical practice. For the design of new antibiotics for this purpose, a total understanding of the structural properties of the enzyme–drug and enzyme–ATP complexes is crucial, which implies a special difficulty because of the size of these complexes. In studies of this nature the 43 or 24 kDa *N*-terminal fragments of B protein from different strains of *Escherichia coli*, have been frequently employed [6,8,11,13,14], but no data are available on the short peptides.

This fact prompted us to consider the possibility of using short synthetic peptides containing segments of GyrB involved in the recognition of coumarins, ATP and DNA as models to carry out interactions



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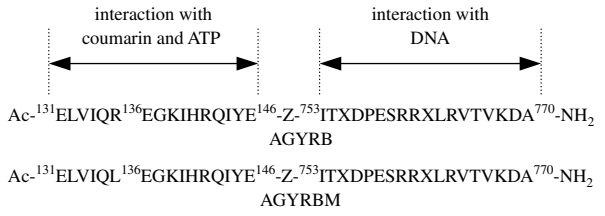

B

Figure 1 Structures of (A) novobiocin and (B) sequences of the peptide mimics of gyrase B protein (X = norleucine and Z = ε-amino caproic acid).

and structural studies. This paper reports a 4.2 kDa synthetic peptide (AGYRB) formed by a *C*-terminal region containing the residues involved in the interaction with DNA [15] and an *N*-terminal region containing the Arg residue at position 136, which is believed to be involved in the coumarin interactions [16] (Figure 1B). Affinity chromatography [17] and fluorescence quenching techniques [18] were exploited to perform the binding studies. Evidence that short peptides might constitute suitable models to study gyrase–coumarin and gyrase–ATP interactions as well as the mechanism of action of the coumarins is presented.

MATERIAL AND METHODS

Chemicals

All chemicals were of analytical grade. 9-fluorenylmethoxycarbonyl (Fmoc)-amino acids were supplied by Novabiochem (San Diego, USA) or Advanced ChemTech (Louisville, USA). 4-(2′,4′-Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido- norleucyl-p-methyl-benzhydrylamine (Rink Amide MBHA) resin (0.55 mmol g^{-1}) was purchased from Novabiochem. Epoxy-activated Sepharose 6B (19–40 μmol ml^{-1}), adenosine 5′-triphosphate-agarose (1.3 μmol ml^{-1}) and single stranded deoxyribonucleic acid-cellulose (3.5 μg g^{-1}) were from Sigma-Aldrich Company. 1-Benzotriazolyloxy-tris-pyrrolidinophosphonium hexafluorophosphate

(pyBOP), 1-hydroxybenzotriazole (HOBt) and 1-hydroxy-7-azabenzotriazole (HOAt) were purchased from Novabiochem. The N,N′-diisopropylethylamine (DIEA) and diisopropylcarbodiimide (DIPCI) were supplied by Fluka Chemical Corp. (USA). Trifluoroacetic acid (TFA) and dichloromethane (DCM) (Sigma-Aldrich USA) were peptide synthesis grade and used directly. Dimethylformamide (DMF), supplied by Mallinckrodt, was bubbled with nitrogen to remove volatile contaminants and kept stored over activated 4Å molecular sieves. N-methyl-2-pyrrolidone (NMP) was purchased from Carlo Erba (Italy). Novobiocin (sodium salt) was also from Sigma-Aldrich.

Substrate DNA

The supercoiled plasmid pBS was prepared in *Escherichia coli* strain DH5α by conventional methods [19] and was purified and stored as described by Marchetto *et al.* [17].

Spectroscopic Measurements

Ultraviolet absorbance and fluorescence measurements were made on a Shimadzu UV-visible 1601PC spectrophotometer and a Varian Cary Eclipse fluorescence spectrophotometer, respectively, both equipped with a thermostatted sample compartment, using a 1.5 ml quartz cell for a magnetic stirrer with a 1.0 cm path length. The fluorescence excitation and emission slit widths were set for 5 nm bandpass, for all measurements.

Peptide Synthesis and Purification

The AGYRB and its mutant version (AGYRBM) whose sequences are reported in Figure 1B were prepared manually, according to solid-phase synthesis methodology using Fmoc chemistry [20] with Rink amide MBHA resin and DIPCI/HOBt or pyBOP/DIEA activation. The functional side chains of Fmoc-amino acids were protected by the following groups: Bu^t for Asp, Glu, Ser, Thr and Tyr, Trt for His and Gln, Pmc for Arg and Boc for Lys. Acetylation was performed with acetic anhydride and DIEA (10 eq each). Peptides were cleaved from the resin by a TFA/water/phenol/thioanisole/1,2-ethanedithiol (82.5 : 5 : 5 : 5 : 2.5) treatment for 2 h at room temperature. The resins were washed with diethyl ether and centrifuged (5×) and the resulting suspension was washed with 10% (AGYRB) or 50% (AGYRBM) aqueous acetic acid. Crude peptides were purified by semipreparative HPLC on a Waters system using a reverse-phase Vydac-C_{18} column (25 × 2.5 cm; 10 μm particles; 300 Å porosity) with a linear gradient of 30%–60% of solvent B (A: water, 0.1% TFA; B: acetonitrile (MeCN) 75% in water, 0.1% TFA) over 90 min. The flow rate was 10 ml min^{-1} and detection was carried out at 220 nm. Analytical HPLC was carried out on a Varian ProStar apparatus employing a Nucleosil C_{18} reverse-phase column (25 × 0.46 cm; 5 μm particles; 300 Å porosity) with a 10%–70% linear gradient of solvent B (A: water, 0.045% TFA; B: MeCN, 0.036% TFA) over 30 min, flow rate 1.0 ml min^{-1} and UV detection at 220 nm. Peptide purity was estimated to be higher than 90% by amino acid analysis (6 M aqueous HCl solution at 110 °C for 72 h) on a Beckman System 6300 analyser. The identity of the peptides was confirmed by electrospray ionization mass spectrometry (ESIMS) on a ZMD model apparatus from Micromass.

Affinity Chromatography

Novobiocin immobilized on Sepharose. Novobiocin was immobilized on epoxy-activated Sepharose as previously reported with ciprofloxacin [17]. 100 mg of novobiocin in 8 ml of 0.3 M carbonate buffer (pH 9.5) was added to 1.2 g of the polymeric support. The mixture was left for 20 h at 37 °C when 0.55 ml of ethanolamine was added and reacted for more than 4 h at 37 °C. After washings with carbonate buffer (pH 9.5), water, acetate buffer (pH 4.0), water, 5 M urea and water, a 1 ml column was packed and equilibrated with standard buffer (10 mM Tris-HCl, pH 7.2/20 mM NaCl/5 mM $MgCl_2$). DNA (pBS; 100 μl, 4.37 μg/μl), peptide (AGYRB or AGYRBM; 500 μl, 20 μM standard buffer solution) or a mixture of both, were added to the column. After 1 h at room temperature and gentle rocking, washings with standard buffer were performed until no absorbance at 260 nm or no fluorescence at 304 nm were detected. The column was washed with 4 M NaCl and equilibrated with standard buffer.

DNA immobilized column. A 1 ml column was packed with a commercial single stranded deoxyribonucleic acid-cellulose and equilibrated with standard buffer (10 mM Tris-HCl, pH 7.2/20 mM NaCl/5 mM $MgCl_2$). AGYRB or AGYRBM (500 μl of a 20 μM standard buffer solution) was left in the column for 1 h at room temperature under gentle rocking. Further washings with standard buffer were performed until no fluorescence at 304 nm was

detected. The column was washed with 4 M NaCl before equilibration.

ATP immobilized column. A 1 ml column packed with adenosine 5′-triphosphate-agarose was employed for this assay. The procedures of equilibrium, washings and elution of AGYRB or AGYRBM were the same as described above for the DNA immobilized column.

Binding Experiments

Novobiocin binding to peptide. The quenching of peptide fluorescence by novobiocin was determined as a function of drug concentration as follows. Aliquots (2–50 μl) from a concentrated novobiocin stock solution (100 μM) were added to a solution of 20 μM of AGYRB or AGYRBM in standard buffer, maintained at 37 °C. Prior to measuring the peptide fluorescence, the samples were homogenized and equilibrated in the cell holder for 6 min with the excitation shutter closed. The excitation wavelength was 280 nm with emission measured at 304 nm. The observed fluorescence intensities were corrected for loss of signal due to dilution effect and for optical filtering effects caused by novobiocin absorption at 280 nm [21]. The data were represented in a Stern-Volmer plot, for static quenching, where the relative peptide fluorescence (F_0/F) was plotted against the novobiocin concentration [NB] whose slope is equal to the association constant (K_a) for complex formation [18], according to Equation (1):

$$F_0/F = 1 + K_a[\mathrm{NB}] \quad (1)$$

In this equation F_0 and F are the fluorescence intensities of peptides in the absence and presence of novobiocin, respectively.

Fluorescence quenching data, obtained by intensity measurements alone, can be explained by either a dynamic or static process. To distinguish them the temperature dependence of quenching analysis was used. The increase in the temperature of analysis results in a decrease in the slope of the Stern-Volmer plots. A careful examination of the absorption spectra of the fluorophore was also employed as an additional method to identify static quenching. In contrast to dynamic quenching, ground state complex formation will frequently result in the perturbation of the absorption spectra of the fluorophore.

ATP binding to peptide. ATP binding was quantified by measuring the decrease in the fluorescence intensity of peptides as a function of ATP concentration. Aliquots (2–50 μl) from a concentrated ATP stock solution (5 mM) were added to a solution of 20 μM of AGYRB or AGYRBM in standard buffer. Experimental conditions such as temperature, equilibrium, fluorescence measurements and corrections as well as data analysis were the same as described above for novobiocin binding.

Peptide binding to DNA. This was carried out using aliquots (5–50 μl) of a concentrated pBS plasmid stock solution (4.37 μg/μl). The quenching of peptide fluorescence was determined as for novobiocin and ATP binding experiments.

Competitiveness Assays

For studies of competitiveness binding of novobiocin and ATP, the peptide model AGYRB (20 μM) in standard buffer was incubated with an equal molar excess of novobiocin for 1 h at 37 °C under gentle rocking. Aliquots (2–50 μl) from a concentrated ATP stock solution (5 mM) were then added to the mixture. Fluorescence was measured (excitation, 280 nm; emission, 304 nm) after the addition of each aliquot as described for the binding assays. Alternatively, AGYRB was incubated for 1 h at 37 °C, with an equal molar excess of ATP. Then aliquots (2–50 μl) of a concentrated novobiocin solution were added and the fluorescence measured at the same excitation and emission wavelength. Prior to all measurements of the peptide fluorescence, the samples were homogenized and equilibrated in the cell holder for 6 min with the excitation shutter closed. The observed fluorescence intensities were also corrected for loss of signal due to a dilution effect and for optical filtering effects caused by novobiocin or ATP absorption at 280 nm. The data were represented in a Stern-Volmer plot and compared with the studies carried out without previous incubation with novobiocin or ATP, respectively.

RESULTS

Peptide Design

Analysis of coumarin-resistant bacterial strains from several species has identified a mutation point to coumarin resistance that maps to the 24 kDa amino-terminal subdomain of GyrB protein [22]. The most prevalent of these are mutations of an arginine residue at position 136 (*E. coli* GyrB). This

latter residue was already implicated in coumarin binding since nine independent coumarin-resistant isolates of *E. coli* have mutations at Arg-136 (to His, Ser, Leu or Cys) [16,23]. The most important contacts anchoring the coumarins in the 24 kDa gyrase domains are located in the region covering the ATP-binding site, including residues around Arg-136. The crystal structure determination of this *N*-terminal subdomain (residues 2–220) complexed with novobiocin [14] as well as with chlorobiocin [13] has revealed that the binding sites of ATP and the drug overlap partially. Nevertheless, it has been proposed that residues surrounding Arg-760 [15] or the corresponding region of the *C*-terminal part of C-TERM [24] are related directly to the recognition and/or transportation of DNA to the DNA binding domain of the A subunit.

According to these findings, a good starting point for the study of the interactions of peptide with coumarin, ATP or DNA, was to consider for peptide design the strand sheet 131–135 and 139–146, the loop residues (136 to 138) that connects them, as well as a short sequence of the native protein containing the Arg-760 residue. Therefore, two fragments of the protein sequence, one including a two-stranded sheet and a loop, and the other including Arg-760 were chosen to be part of the primary structure of the peptide model. Their lengths were set according to the presence of basic residues in the corresponding natural sequences. Finally, to simplify the synthetic problems, a flexible linker replaced the 607 amino acid native sequence that connects these two fragments. Taking into account all these considerations, the model peptide AGYRB was built using the natural fragments 131–146 and 753–770, and a residue of ε-amino caproic acid (Z) to connect them (Figure 1B).

Resistance to coumarin drugs seems to be a consequence of the loss of drug binding to the gyrase induced by mutations in the enzymatic sequence. According to the literature, the amino acid usually mutated in spontaneous coumarin-resistance strains is Arg-136 of GyrB [9,16,22,23]. In particular, mutations of Arg-136 to Leu or to His induce high levels of resistance to coumermycin A_1 [25] and novobiocin [26], respectively. These observations prompted us to consider a comparative study between AGYRB and its analogue AGYRBM, the peptide that mimics the Leu-136 mutant GyrB (Figure 1B). Both peptides were acetylated at the *N*-terminus and have a carboxamide group at the *C*-terminus in order to mimic the presence of amide bonds at these positions in the protein. In order to prevent undesired oxidation and/or alkylation processes during the synthesis of the peptides, Met residues were replaced by norleucine. In addition, it was decided not to include any fluorescent probe on the peptides, basically to maintain the primary structure as close as possible to the structural features of coumarin, ATP and DNA binding sites in the gyrase B protein.

The peptides AGYRB and AGYRBM were synthesized and purified by solid phase synthesis, as described in the material and methods. After purification, 27.3 mg of AGYRB (15% overall yield) and 20.6 mg of AGYRBM (12% overall yield) were obtained. HPLC: rt, 16.1 min and 21.1 min to AGYRB and AGYRBM, respectively. Electrospray ionization mass spectrometry (ESIMS): m/z $(M + 2H)^{2+} = 2115$ (AGYRB) and 2093 (AGYRBM); $M_T = 4229.7$ and 4186.2, respectively.

The objective of this study was to determine whether novobiocin or ATP could form a stable complex with AGYRB, as well as whether this complex is stabilized by the presence of DNA. Furthermore, the possibility of the formation of a similar complex, stabilized or not by DNA, with AGYRBM, is also part of this research. For this purpose, assays based on affinity chromatography with novobiocin, ATP and DNA covalently anchored to the resins were designed.

Novobiocin Affinity Chromatography

With the purpose of obtaining evidence on the formation of a complex between novobiocin and the peptide model AGYRB, the first assay was performed using the coumarin bound to the polymeric support. To this aim epoxy-activated Sepharose was utilized, to which the drug was anchored by its nucleophilic phenolic hydroxy group of the 4-hydroxy-3-(3-methyl-2-butenyl)-benzoic acid moiety under weakly alkaline conditions (Figure 2).

The peptides and DNA were detected following the fluorescence emission at 304 nm (excitation at 280 nm) and the UV absorption at 260 nm, respectively. The chromatographic behaviour of peptide and peptide/DNA using the drug-immobilized column are shown in Figure 3 (A and B). In the presence of magnesium and in the absence of DNA, AGYRB had affinity for the novobiocin column (Figure 3A), but when a mixture of the peptide and DNA was employed, the retention was amplified (Figure 3B), as demonstrated by their elution when the ionic strength was increased. On the other hand,

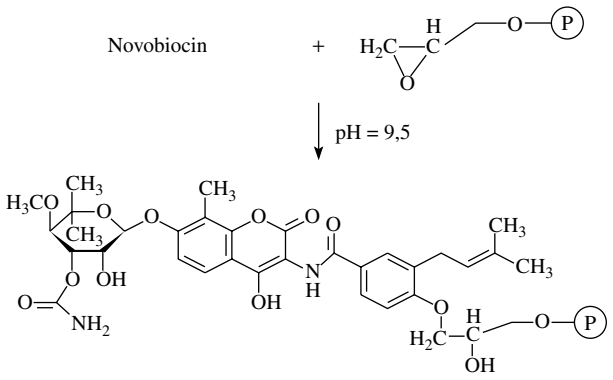

Figure 2 Anchorage of novobiocin to Sepharose.

the peptide mimic of the mutant GyrB (AGYRBM) different to AGYRB was retained in the column only in the presence of DNA. DNA retention was observed in experiments performed in our laboratory under the same conditions and with the column mentioned above (results not shown). All results were dependent on the presence of Mg^{2+}. The peptides and DNA were unable to bind to the resin in a control experiment carried out using unmodified Sepharose.

DNA Affinity Chromatography

The interaction of peptides with DNA was evaluated by the retention in a DNA-cellulose column. Figure 3C shows the results concerning the affinity of peptides to the DNA-resin. The peptides were detected following the fluorescence emission at 304 nm (excitation at 280 nm) as already described. Unlike the study carried out with the drug anchored to a resin, the fact that using DNA bound to a polymeric support allowed us to determine

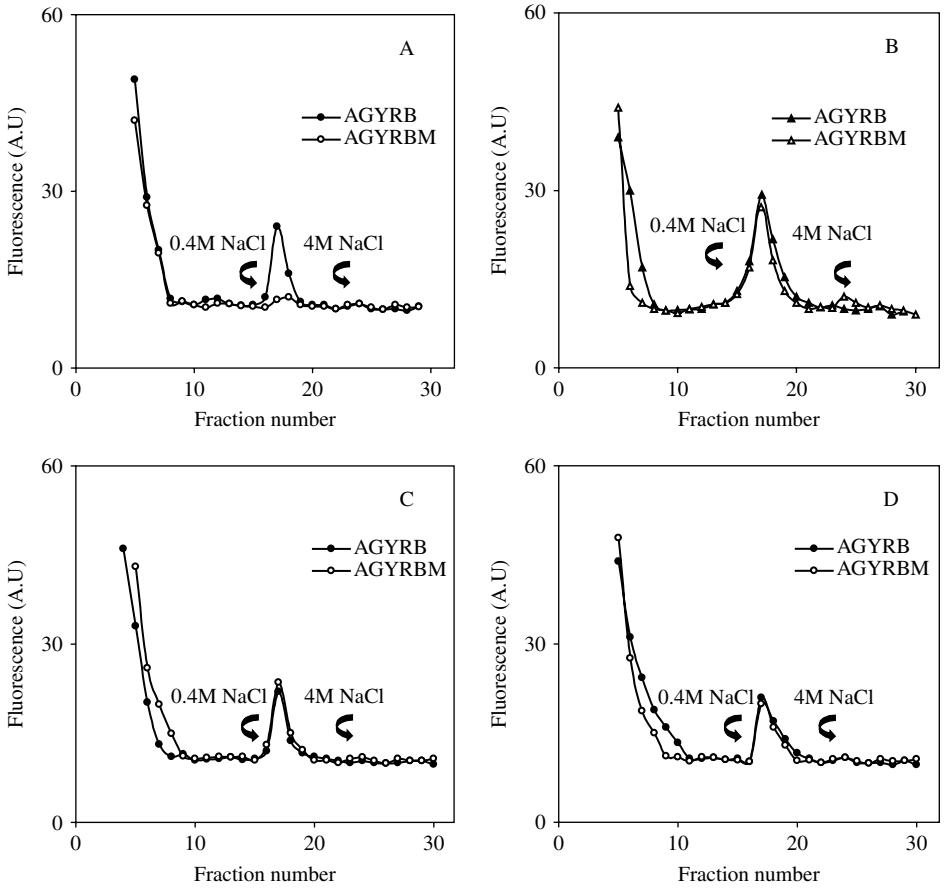

Figure 3 Affinity chromatography experiments with novobiocin (A and B), DNA (C) and ATP (D) bound to the polymeric support. Samples: peptide alone (A, C and D); peptide-DNA (B). The columns were equilibrated with the buffer 5 mM Tris.HCl, pH 7.2/20 mM NaCl/5 mM $MgCl_2$. Elution was carried out with the same buffer containing 0.4 or 4 M NaCl as indicated. The peptide elution was monitored by the intrinsic fluorescence of peptide mimics (excitation at 280 nm and emission at 304 nm). Fractions having fluorescence values out of scale are not presented. AU = Arbitrary units.

qualitatively whether the peptides were able to interact with DNA.

As indicated by the elution profiles, both AGYRB and AGYRBM were retained in the column in a similar way, but the presence of magnesium ions was also essential. Although in the assay conditions used, the effective charge of the peptides was not similar, no difference between the different complexes was observed in terms of stabilization by electrostatic interactions (both were eluted with 0.4 M NaCl). No retention of peptide was observed in a series of control assays that were performed using unmodified resin.

ATP Affinity Chromatography

The binding of the peptides to ATP was assessed using an adenosine 5′-triphosphate affinity column. According to the chromatographic profiles shown in Figure 3D, both peptides, AGYRB and AGYRBM, were retained in the column and were eluted with 0.4 M NaCl. Thus ATP binds, apparently with a similar affinity, to both AGYRB and AGYRBM. With regard to magnesium ions, no appreciable affinity between the peptides and the column with ATP immobilized was observed when the experiments were carried out in the absence of this ion. Control assays carried out using unmodified resin showed no retention of peptides to the column.

Binding Studies

The experiments of affinity chromatography revealed interactions among the peptide mimic AGYRB and novobiocin or ATP. The interesting point was that the interactions were independent of the presence of DNA. However, this behaviour was not observed in the case of the peptide mimic of the mutant GyrB (AGYRBM). This result prompted us to determine the binding parameters for the systems mentioned above. The binding parameters were studied by following the quenching of the intrinsic fluorescence of the peptide upon binding of the novobiocin, ATP or DNA [27]. The plot of the relative peptide fluorescence intensity at an emission wavelength of 304 nm ($\lambda_{ex} = 280$ nm) as a function of total novobiocin, ATP or DNA, is shown in Figure 4. The association constant (K_a) values shown in the Table 1 were obtained from the slope of the Stern-Volmer plot for identified static quenching [18], according to Equation (1). The reported values of

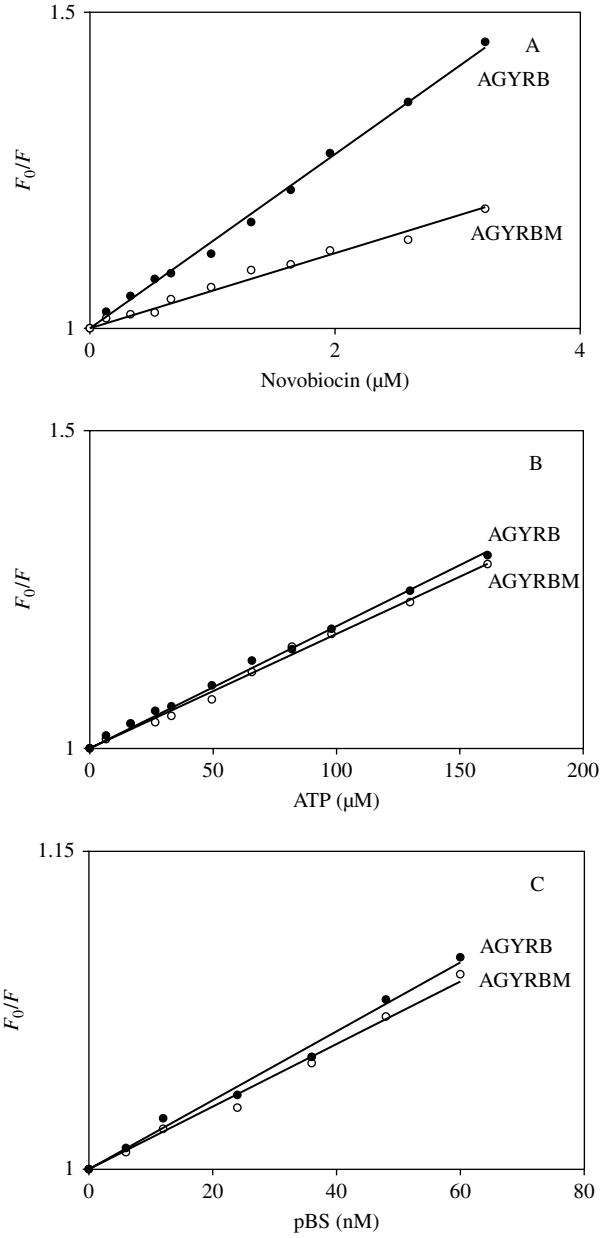

Figure 4 Binding determinations using Stern-Volmer plots. (A) Peptide (30 nmol) and novobiocin (0.13–3.23 μM). (B) Peptide (30 nmol) and ATP (6.65–161.30 μM). (C) Peptide (30 nmol) and pBS (6–60 nM). The slope of the linear Stern-Volmer plot represents the association constant for static quenching. For dynamic quenching the slope represents the Stern-Volmer quenching constant.

K_a are the means of at least five measurements with the standard deviation.

As already found using affinity chromatography, novobiocin and ATP bind to AGYRB in the presence of Mg^{2+}. A preferential binding of the drug to

Table 1 Comparative Binding[a]

Entry	Ligand	$K_a(\times 10^3\ M^{-1})$		$K_d(\mu M)$	
		AGYRB	AGYRBM	AGYRB	AGYRBM
a	Novobiocin	140 ± 30	—[b]	7.1 ± 1.2	—[b]
b	ATP	1.9 ± 0.4	1.7 ± 0.3	530 ± 95	590 ± 90
c	pBS	1600 ± 200	1400 ± 100	0.63 ± 0.08	0.71 ± 0.07

[a] Fluorescence binding assays were performed as described in the Material and Methods section. Reaction mixtures contained 20 μM of one of the peptides and various amounts of ligand. The association constant (K_a) was determined by Stern-Volmer plots [18]. The values of the dissociation constant, K_d ($=1/K_a$), also are shown.
[b] Quenching identified as dynamic quenching, therefore no binding was observed.

peptide was observed in this case (about 70 fold in relation to ATP, entry 'a' and 'b' of Table 1). On the other hand, no binding of novobiocin to the peptide mimic AGYRBM was observed (entry 'a' of Table 1), however, the binding of ATP was identical to both AGYRB and AGYRBM (entry 'b' of Table 1), which is in agreement with our observations using affinity chromatography.

Regarding the interaction of peptides with DNA, the results of binding studies (entry 'c' of Table 1) were consistent with the affinity chromatography studies. Both peptides, with invariable *C*-terminal fragment 753–770, showed identical interactions with DNA. In addition, no change in the binding parameters was observed when the binding assays were carried out with mixtures containing novobiocin or ATP (data not shown).

Competitiveness Assays

To address the question of whether coumarin binds to the peptide mimic AGYRB competitively with ATP, as described for gyrase [14], the binding of novobiocin and ATP to the AGYRB peptide (Figure 5) was examined by fluorescence quenching. When the AGYRB peptide was pre-incubated with ATP and novobiocin was subsequently added for fluorescence quenching analysis, no change in the Stern-Volmer plot was observed when compared with the plot obtained without ATP pre-incubation (Figure 5A). Consequently the association constants were apparently the same for both cases ($1.4 \times 10^5\ M^{-1}$), suggesting that ATP does not affect novobiocin binding. On the other hand, when AGYRB was pre-incubated with novobiocin and ATP was subsequently added for the fluorescence intensity analysis, a lower slope of the Stern-Volmer plot was observed and the association constant was smaller than the corresponding value, obtained without pre-incubation with novobiocin (Figure 5B).

DISCUSSION

The results presented above with peptides designed as mimics of GyrB (wild type and mutant) confirmed important features already known about the mechanism of action of coumarins. In this work, similar conclusions can be drawn from the results achieved with both affinity chromatography and fluorescence quenching methods.

It is well established that the intracellular target of the coumarin group of antibiotics is DNA gyrase and that these compounds inhibit the supercoiling and ATPase reactions of gyrase [28,29]. It is known that residue Arg-136 of the gyrase is a key interaction in terms of the stability of the protein–coumarin complex [16,26], and also that the ATP and coumarin-binding sites are in close proximity [9,14]. Also, it is clear that the presence of DNA is not required for ATP binding [29] but the binding of DNA to B protein is essential for ATP hydrolysis [30]. On the other hand, the DNA dependence of the coumarin interactions remains unclear.

From a qualitative point of view, a parallel behaviour was found for AGYRB, the peptide mimic of GyrB. Thus, AGYRB is able to bind novobiocin and ATP, independently of the DNA presence (Figure 3A,D), consistent with the notion that the *N*-terminal part of the B protein, including Arg-136 residue is concerned with novobiocin and ATP interactions and DNA is not required in order to promote these interactions. The retention increase of the AGYRB on the coumarin-immobilized column in the presence of DNA (Figure 3B) is probably due

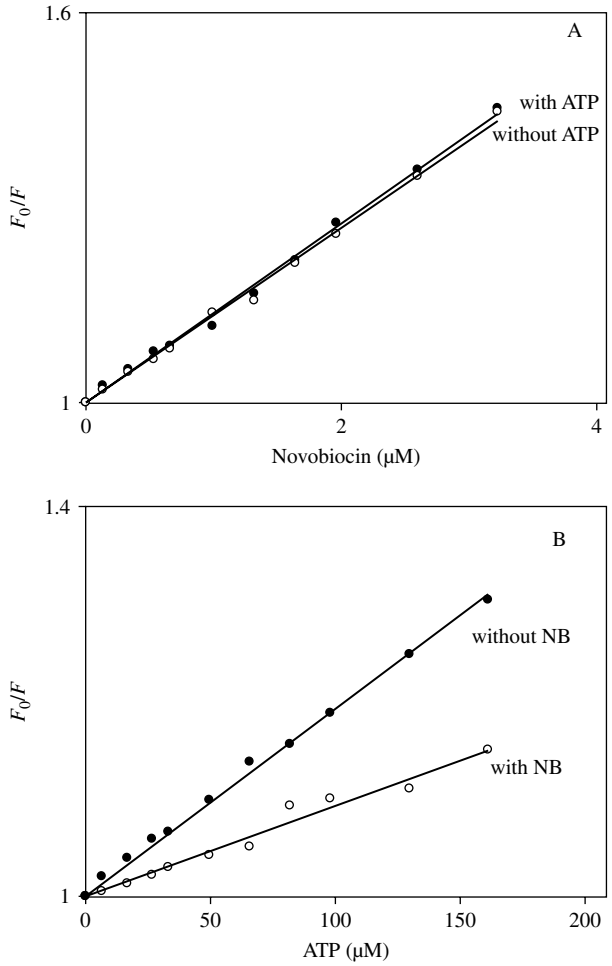

Figure 5 The binding of novobiocin (A) and ATP (B) to the AGYRB peptide. The peptide (20 μM) was incubated with an equal molar excess of ATP for 1 h and novobiocin (0.13–3.23 μM) added (A). The peptide (20 μM) was incubated with an equal molar excess of novobiocin (NB) for 1 h and ATP (6.65–161.30 μM) added (B). Fluorescence was measured (excitation, 280 nm; emission, 304 nm) after each aliquot addition. The slope of the linear Stern-Volmer plot represents the association constant for static quenching.

to simultaneous retention of both the AGYRB and the AGYRB-DNA complex, components of the sample added to the column, in agreement with the binding parameters. As already reported in the literature for B protein, Mg^{2+} was required in order to promote the interactions among the involved species [8,31].

The elution of AGYRB from the novobiocin affinity column by mild conditions suggests that hydrogen bonds are the principal determinants of peptide–drug binding. The importance of Arg-136 might suggest an ionic interaction between this residue and the drug, but in the experimental conditions the drug was unable to interact by this type of interaction due to the absence of a negative effective charge in its structure. Studies carried out with the 24 kDa protein, also suggested that ionic interactions are not likely to be important for drug binding [32]. The Mg^{2+} ion plays an important role in peptide–drug interactions and it can be coordinated with six water molecules, so it is possible that the novobiocin interacts with AGYRB through hydrogen bonds, with Mg^{2+} acting as bridge between the guanidinium group of Arg-136 and the lactone coumarin ring of the drug. The interaction of the AGYRB with the drug produced a quenching in the peptide fluorescence, identified by temperature dependence and absorption spectra analysis as static quenching. Thus, the association constant for the peptide–drug complex was 1.4×10^5 M^{-1}, lower than that reported for 24 and 43 kDa DNA gyrase fragments ($K_a \approx 10^7$ M^{-1}) [8,13,26], which is not surprising considering the size of these fragments (6 and 10-fold bigger, respectively).

In contrast to the novobiocin–AGYRB complex, in the ATP binding electrostatic interactions could be involved. In this case, the phosphate group binds by ionic interactions with Mg^{2+} and the water molecules coordinated to this ion interact with the peptide by hydrogen bonds. ATP binding could also be stabilized by other interactions considering that the adenine rings could make a number of polar contacts with the peptide, in particular with Tyr-145 and the side chain of the glutamic acid residues. The affinity chromatography elution conditions already suggested a weak ATP binding, which was confirmed by the association constant of the ATP–AGYRB complex (1.9×10^3 M^{-1}). Of interest is the fact that the association constant for gyrase is two orders of magnitude higher than the peptide [33], the same difference as observed for the complexes of the peptide or protein with the drug. In addition, the binding of novobiocin to AGYRB is apparently much tighter than for ATP; the K_a for ATP is $\approx 10^3$ M^{-1} compared with 10^5 M^{-1} for novobiocin, in agreement with data described by other authors [3,33,34] for DNA gyrase ($\approx 10^5$ M^{-1} for nucleotides and 10^7–10^9 M^{-1} for coumarins).

According to the K_a values for the AGYRB–DNA, AGYRB–novobiocin and AGYRB–ATP complexes, the peptide binds to DNA more efficiently than the other species, which could be explained in terms of the nature of the interactions that are responsible for binding. The presence of several basic residues in AGYRB is probably the key feature for the binding of

the peptide to DNA through electrostatic interactions between these residues and the phosphate groups of the nucleic acid [17,35]. However, the Mg^{2+} ion dependence observed seems to indicate that the peptide interacts with DNA through its acidic residues within the 753–770 *C*-terminal fragment of AGYRB, to form a complex in which Mg^{2+} acts as a bridge between the phosphate groups of DNA and the peptide, as proposed recently for gyrase [31]. On the other hand, the affinity of AGYRB for DNA ($\kappa_a \approx 10^6$ M^{-1} for pBS) proved to be much lower than that reported for DNA gyrase ($\kappa_a \approx 10^{10}$ M^{-1}) [36], which is not surprising if the size of the natural protein is considered.

The binding of the peptide mimic of mutated GyrB, AGYRBM, to DNA proved to have the same efficiency as AGYRB (Figure 3C and Figure 4C). The change in the effective charge of the peptide from +1 to 0 as a consequence of the mutation of Arg to Leu at position 136 was unable to promote any alteration in the affinity, even with the reduction of the electrostatic interactions with negatively charged phosphate groups. This suggests that the common *C*-terminal fragment in the peptides is concerned with the DNA binding, consistent with the 43 kDa *N*-terminal B fragment that was unable to bind to the DNA [6].

Unlike AGYRB, novobiocin did not bind to AGYRBM, as revealed by the affinity chromatography and fluorescence quenching techniques (Figure 3A, entry 'a' of Table 1). This behaviour is in agreement with several experimental data [22,23,26,36] for the novobiocin and gyrase B fragments with a mutation in Arg-136, that confers coumarin resistance. In those cases, the gyrase B fragments showed greatly reduced drug binding. The retention of the mutated peptide AGYRBM in the novobiocin column in the presence of DNA is additional evidence for the involvement of the *C*-terminal fragment of the peptides with the DNA binding. In this case, the AGYRBM-DNA complex was retained but not the AGYRBM. As with AGYRB, a decrease in the fluorescence intensity of the peptide as a function of the drug concentration was observed (Figure 4A), but the temperature dependence and absorption spectra analysis identified that the quenching was dynamic quenching, resulting from collisional encounters between species and was not due to complex formation. Therefore, the slope of the Stern-Volmer plot is not equal to the association constant, but just to the quenching constant. The reduced affinity displayed by the mutant peptide suggests that the loss of the hydrogen bond between Arg-136 and the drug results in a smaller binding, presumably because of the absence of this important interaction when this residue is changed to Leu. On the other hand, the mutation at the critical position 136 did not produce any change in the ATP binding (Figure 3D and Figure 4B, entry 'b' of the Table 1). This implies that possibly this residue is not a part of the ATP binding site. In fact, a number of the mutations that confer coumarin resistance to DNA gyrase [23,25] lie at the periphery of the ATP binding site.

Many studies reported in the literature have suggested that the ATPase activity of DNA gyrase is inhibited in a competitive manner by novobiocin [3,11,34]. If so, according to the results of competitiveness, the use of short peptides such as AGYRB could be a suitable approach to evaluate the competitive nature of the inhibition of the gyrase by novobiocin. The results of competitiveness assays indicate that, within the limits of experimental error, novobiocin prevents ATP binding, indicating overlapping binding sites, supporting the idea that the novobiocin is competitive with ATP. This conclusion is consistent with x-ray crystallography data on the structure of the complex between the 24 kDa protein and novobiocin [14].

In conclusion, this study has shown that the use of short peptides including sequences of GyrB provides an alternative way to study and better understand the key contacts between the coumarins or ATP and the enzyme, which are especially important for a rational drug design. Moreover, it is believed that these studies provide additional proof that the model of inhibition of coumarin drugs is competitive and that the ATP and novobiocin binding sites are not the same but are in close proximity. Thus, AGYRB constitutes a starting point for the development of peptide models able to mimic the structural features of GyrB at the coumarin and ATP binding sites and, it can be used in the search for new powerful and more specific inhibitors of DNA gyrase.

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