PhD thesis

Investigation of MreB as bacterial actin by biophysical methods

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INTRODUCTION

MreB is a prokaryotic actin protein, named after murein, the major component of the cell wall (Mre, i.e. Murein Region E). It is found in almost all non-coccoid bacteria. It is present in both Gram-positive and Gram-negative bacteria, although the *mreB* gene is more abundant in Gram-positive organisms[1]. A chromosomally encoded protein located at the mre locus. The eukaryotic actin orthologues MreB filaments directly regulate cell shape, play a role in morphogenesis [2, 3], cell division, cell wall synthesis, establishment of cell polarity, protein localization, cell organelle localization, in addition to chromosome segregation and replication [4, 5]. Polymerisation experiments based on light scattering have shown that MreB proteins can polymerise in the presence of ATP, AMP-PNP, GTP, or ADP [6-9]. In the case of *Bacillus subtilis*, nucleotide-independent polymerisation has also been observed [8].

The MreB literature can be grouped according to the buffer conditions used. There are research groups that performed the experiments in a salt-free buffer [6, 8, 9], while others worked with renatured protein in a high-salt buffer and induced polymerisation by adding nucleotides [7, 10-12]. Like actin, MreB can also be an ATPase, because in most cases it requires ATP for polymerisation, and polymerisation can facilitate the hydrolysis of nucleotides [13]. The monomers bind ATP, which hydrolyses to ADP and Pi (inorganic phosphate) during its polymerisation, and then cleaves the inorganic phosphate. Finally, ADP is replaced by ATP and the process begins again. However, how this process takes place in the case of MreB is not yet clear. What is certain is that this ATPase activity and phosphate release have also been proven in the case of the MreB protein from several bacterial species. In addition, nucleotide binding during the polymerisation of MreB affects the critical concentration. Similarly, temperature increases the critical concentration, and at higher temperatures the polymerisation of MreB will be faster [6]. In the case of eukaryotic actin, it was described that a closed (in the case of a polymer) and an open (monomer-specific) conformation are distinguished during polymerisation [14]. During the polymerisation, the MreB filament of *Leptospira interrogans* becomes more closed, and in the first step the decrease in fluorescence intensity cannot be followed by light scattering experiments. The second step is slower, after the conformational transition of the monomers, the formation of MreB filaments and superstructures takes place. Molecular dynamics simulations support the hypothesis that the conformational changes of *Thermotoga maritima* MreB (*Tm*-MreB) are similar [13].

In order to investigate the structural changes of the filaments formed during polymerisation, Colavin and his research group [13] used simulation measurements to demonstrate that MreB exhibits actin-like, polymerisation-dependent structural changes in which the MreB subunits

flatten during polymerisation, which alters the nucleotide binding pocket in such a way as to facilitate the hydrolysis. A nucleotide-dependent "bending" was observed between the subunits of the MreB filaments, and the hydrolysed polymers resulted in a stiffer conformation. Their simulations suggest that this mixed population of structures, i.e., straight, ADP-bound and bent, ATP-bound filaments, represent the two different nucleotide states. Ultimately then, hydrolysis may lead to a straightening of the filaments [13]. Their results suggest that the state of MreB hydrolysis should also be considered; thus, when a curved, ATP-bound MreB filament binds to the relatively flat membrane, the filament may straighten, which in turn may promote hydrolysis or preferential binding of MreB to specific regions of membrane curvature [15]. Nucleotide status can also affect the affinity of MreB for other proteins, as has been observed for actin and actin-binding proteins [16].

Most proteins adopt a specific three-dimensional structure. By examining the thermal stability of the protein, we can find out how stable the structure is and how different environmental factors affect it. In the case of thermophilic, i.e. heat-tolerant species, there may be several reasons for higher temperature stability at the protein level, such as greater hydrophobicity, shortening of loop-like structural elements ("loops"), oligomerisation (monomers do not form long filaments, the polymerisation process gets stuck at a lower level), free surfaces covered during the process, amino acid exchange in the secondary structure, higher abundance of proline derivatives, lower abundance of heat-sensitive amino acids, stronger hydrogen bonds, presence of salt bridges [17]. Salt bridges stabilize the structure of the protein so that it can resist external influences, melting, or unfolding at higher temperatures. Salt bridges that are close to each other work together, reinforcing each other and increasing stability. Salt bridges can hold protein structures together and stiffen them [17]. As a result of thermal denaturation, the protein structure undergoes changes. In general, the internal fluorophores in the protein become more exposed to environmental effects, which typically means a change in tryptophan fluorescence quantum efficiency (the ratio of emitted and absorbed photons) and a shift in the fluorescence spectrum to longer wavelengths. For this reason, the process of heat denaturation can be easily followed by measuring the fluorescence spectrum of tryptophan. A decrease in fluorescence intensity or a red shift of the spectrum clearly indicates a structural change due to increased heat [18]. The *Thermotoga maritima* MreB we studied contains a single tryptophan amino acid located in the central part of the molecule, between two subdomains. Accordingly, with the specific excitation of tryptophan in the spectral changes that occur under the influence of heat, we can obtain information mainly from this part of the molecule.

Antibiotics exerts their effect on bacteria in different ways and have different targets. They can be bactericidal, which means they kill the bacteria, or have a bacteriostatic effect, e.g., they only inhibit the formation of the nascent cell wall, but have no effect on the cell wall already formed, i.e., only prevent proliferation. Depending on which cellular processes they inhibit, they can be divided into six broad groups: antibiotics that inhibit cell wall synthesis, inhibitors of protein biosynthesis, inhibitors of membrane function, antibiotics that inhibit nucleic acid synthesis, inhibitors of metabolic pathways, antibiotics that inhibit ATP synthase [19]. Vancomycin is a glycopeptide that is usually used as a last resort antibiotic to treat Gram-positive infections. Its target is the cell wall, more precisely the membrane-bound lipid II molecule, which is the precursor of bacterial peptidoglycan biosynthesis. Vancomycin binds with its N-terminal part to the D-Ala-D-Ala region at the C-terminus of the lipid II. The structure of vancomycin bound to the tripeptide PG analogue was described in the early 1980s. The structure shows that the D-Ala-D-Ala strain of PG binds to the cleft formed by a vancomycin aglycone (a non-sugar component of the molecule) and is stabilized by five H-bonds [20]. Vancomycin is a large hydrophilic molecule that is not transported on membrane porin molecules and cannot freely diffuse across the outer membrane [21], making it ineffective against Gram-negative bacteria because it cannot enter the cell. With a fluorescently labelled antibiotic, it is easier to follow the mechanism of action of the antibiotic within the cell or even within the living organism [22]. BODIPY-vancomycin and other fluorescent derivatives are used to hydrolyse peptidoglycan biosynthesis in *Bacillus subtilis*. However, the minimum inhibitory concentration (MIC: the smallest amount of antibiotic in ml that inhibits the growth of all bacteria) of fluorescent vancomycin is higher than that of the conventional form, meaning it is less effective. The negatively charged fluorophore prevents the complex form penetrating the anionic peptidoglycan layer of the bacterium. This is usually remedied by introducing the unlabelled antibiotic into the bacteria in addition to the fluorescently labelled antibiotic [23]. However, due to its relatively small size and neutrality, BODIPY did not increase the minimum inhibitory concentration as much as the fluorescein-labelled antibiotic [23].

The other antimicrobial compound we studied is A22, which acts directly on the MreB protein and prevents bacterial cell growth and proliferation by blocking it. A22 (S-(3,4-dichlorobenzyl)isothiourea) is an S-benzylisothiourea derivative [24, 25]. A22 binds to MreB with micromolar sensitivity (minimum inhibitory concentration: ~13 μg/ml) and interferes with the polymerisation process [26]. It also inhibits bacterial motility, adhesion to surfaces, and biofilm formation - properties necessary for the development of bacterial infections and antibiotic resistance. [27, 28]. In molecular dynamics simulations, Awuni et al. found that A22

binds to the groove next to the nucleotide binding pocket of MreB [29] and its binding affinity is 20-30 times higher in the presence of di- and trinucleotide phosphates [25]. In the case of ATP-A22-MreB, A22 interacts with the γ-phosphate group of ATP via a hydrogen bond, and this interaction prevents or slows the cleavage of the γ-phosphate, leading to filament instability. In their view, A22 prevents the ATP-initiated structural changes required for the formation of a stable MreB polymer structure. [29]. Literature data indicate that as a result of treatment with A22, the helical localization of MreB *in vivo* within the cell ceases, it is depolymerised causing MreB to become diffusely localised in the cytoplasm and lose its rod shape and viability [4, 26, 30]. A22 has a bacteriostatic effect. In the case of *Caulobacter crescentus* species, A22 at a concentration of 10 μg/ml (~50 μM) slowed the growth of cells, which assumed a spherical shape under the influence of the drug. However, a concentration of 100 μg/ml already stopped cell growth, but did not cause changes in cell shape [30]. A22 inhibits biofilm formation, which contributes significantly to the infectivity of the bacteria. It can be assumed that compound A22 acts in the initial phase of adhesion and affects the basic parameters of biofilm development, such as the motility of bacteria and adhesion to surfaces. Since compound A22 alters cell shape by acting on the MreB protein of the bacterial cell wall, it is likely that it can also destabilize or dissolve parts of the cell such as flagella and fimbriae, which affects bacterial motility [27]. Therefore, it stands to reason that A22 or its more potent derivatives should be used as antibiotics for therapeutic purposes based on its mechanism of action. This is confirmed by the fact that it is neither cytotoxic nor genotoxic, i.e., it does not attack cells or genetic material. Since it acts directly on MreB, it can be a new antimicrobial agent against multiresistant bacteria [31].

AIMS OF THE THESIS

Based on the previously described MreB literature, it is clear that the *in vitro* examination of MreB proteins faces serious difficulties, since the purification of the protein and keeping it in a stable, functional state is a great challenge [6, 11, 32].

We set out to solve this, to be able to produce functional protein in large quantities under native conditions. For this, we used a new bacterial expression system (*E. coli* BL21 Arctic Express (DE3)). This expression system also contains chaperone proteins, and the expression takes place at a lower temperature, which can help the protein adopt its native conformation and prevent the formation of inclusion bodies. We continued our experiments with the MreB protein of *Thermotoga maritima*, one of the most common bacterial species described in the MreB literature. The monomer crystal structure of this MreB was first described [10], in addition, it also contains a tryptophan amino acid that can be easily examined from a spectroscopic point of view.

In the second half of my dissertation, we investigated the effects of A22 and vancomycin on the *Tm*-MreB protein produced as described above. In our experiments, we also examined the interaction of the two antibiotics.

- Our aim was to develop a native discovery protocol for *Thermotoga maritima* MreB, in a newly applied ArcticExpress (DE3) expression system, which had not been used for MreB proteins before.
- Functional tests of the purified *Tm*-MreB followed. For this, we primarily performed denaturation tests: we obtained information on the thermal and chemical stability of *Tm*-MreB by the method of heat denaturation and chemical denaturation, and by monitoring tryptophan emission.
- Our goal was to determine the MreB nucleotide binding and the effect of different nucleotides on protein polymerisation.
- Among our plans was the description of the binding of A22 and vancomycin to *Tm*-MreB, and the characterization of their effect on the protein.
- We wondered how A22 changes the localization of MreB inside the cell.
- We also aimed to study the effect of the two antimicrobial agents on the growth of *E. coli* bacteria cells. For this, we designed reproduction tests, which we supported with microbiological methods.

MATERIALS AND METHODS

Thermotoga maritima MreB protein native preparation

The plasmid containing *Thermotoga maritima* MreB was transformed into ArcticExpress (DE3) (Agilent Technologies) competent cells. 2 µl of ß-mercaptoethanol diluted with distilled water at a ratio of 1:10 was added to 100 µl of competent cells. Cells were incubated on ice for 10 min, rotating the tubes every 2 min. After 10 minutes, 5 µl of plasmid DNA was added to the competent cells. Transformed cells were then incubated on ice for 30 min. After a 20-second heat shock (42 °C water bath) the cells were placed on ice again for 2 minutes. The transformed bacterial cells were grown in 0.9 ml of pre-warmed Luria Broth (LB) nutrient solution at 37 °C for 1 hour, shaking at 220-250 rpm. Then, 150 µl of cell culture was spread on a plate containing double resistance (gentamicin: 20 µg/ml and ampicillin: 100 µg/ml).

A separate colony was inoculated with 100 ml of LB solution containing the appropriate amount of both antibiotics. The cell culture was grown overnight in a shaking incubator at 37 °C (220-250 rpm). The next day, 20-20 ml of cell culture was added to 1-1 l of LB nutrient solution and shaken at 30 °C at 220-250 rpm for 3 hours. After the incubation period, the temperature was taken back to 11.5 °C. As the cells cooled back to 20°C, they were expressed with IPTG at a final concentration of 0.8 mM for 24 h. The cells were centrifuged (2,900xg, 10 min.) and the pellet was stored at −20 °C.

The *Thermotoga maritima* MreB cell pellet (1-2 g) was homogenized in TRIS-HCl buffer (50 mM TRIS, pH 8.0, 1 g pellet/10 ml buffer). After adding lysozyme, the cells were sonicated on ice (80%, pulse for 1 minute, then 1-minute break, repeated 6-7 times). DNase I (50 µg/ml) was added to the lysate before centrifugation (328,000xg, 4 °C, 30 min). The Ni-nitrilotriacetic acid (Ni-NTA) (Qiagen) column was eluted with 5% imidazole buffer (1M imidazole, 50 mM TRIS-HCl, pH 6.0) and 95% NaCl buffer (300 mM NaCl, 50 mM TRIS-HCl, pH 6.0). equilibrated and the supernatant was cooled and stirred for at least 1 hour. The column was then washed with 10-20-30-50% imidazole buffer (dissolved in NaCl buffer). The fractions were collected separately and analysed by SDS gel electrophoresis.

Fractions containing MreB were dialyzed in buffer A (4 mM TRIS-HCl, 0.1 mM $CaCl_2$, pH 7.5) with two buffer exchanges. MreB was finally ultracentrifuged (328,000xg at 4°C for 30 min). The His tag was removed with PreScission protease (GE Healthcare Life Sciences) (2U/100 µg protein), which was left beside the protein overnight at 4 °C. The protease was removed next day on a glutathione column, thanks to the GST tag of the protease. The final protein concentration was measured with a spectrophotometer. Extinction coefficient: 11,460 $M^{-1}cm^{-1}$.

Functionality tests

Light scattering measurements

The polymerisation of MreB was followed by light scattering measurements with a SAFAS Xenius XLS Fluorimeter, setting the excitation and emission wavelengths to 400 nm. To investigate the polymerisation processes of MreB, a concentration of 100 mM KCl was used (P100: 4 mM TRIS, 100 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA). In our experiments, A22 was added to the protein solution at the beginning of the polymerisation, at the same time as the polymerisation salt. We hypothesized that the more filaments are formed or are present in the solution, the greater the degree of scattering.

Inorganic phosphate measurement

EnzCheck® Pyrophosphate Assay Kit (Biocenter) was used to measure the phosphate produced during MreB ATP utilization. In the presence of inorganic phosphate, the enzyme 2-amino-6-mercapto-7-methylpurine ribonucleotide (MESG) decomposes into ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine with the help of purine nucleoside phosphorylase (PNP). During this enzymatic transformation, the absorption maximum of 2-amino-6-mercapto-7-methylpurine shifts from 330 nm to 360 nm. Followed over time, the increasing optical density at 360 nm is directly proportional to the amount of released inorganic phosphate.

Tryptophan fluorescence measurements

20 μM *Tm*-MreB or 20 μM tryptophan amino acids were measured in salt-free (4 mM TRIS-HCl, 0.1 mM $CaCl_2$, pH 7.5) or high-salt (20 mM TRIS-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.5) buffer, with a Horiba Jobin Yvon spectrofluorometer. The excitation was set to 295 nm, the emission was measured between 310 nm and 450 nm at 22 °C.

Temperature-dependent tryptophan emission measurement

20 μM *Tm*-MreB protein was tested under two buffer conditions in the presence or absence of 2 mM ATP. Using salt-free buffer (4 mM TRIS-HCl, 0.1 mM $CaCl_2$, pH 7.5) or high-salt buffer (20 mM TRIS-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.5) the protein was heated from 20 °C to 95 °C and the fluorescence intensity of tryptophan was continuously measured between 310-450 nm. The excitation wavelength was 295 nm. The measurements were performed on a Jobin Yvon Horiba fluorimeter equipped with a Quantum Northwest TLC50 temperature-controlled cuvette holder.

Chemical denaturation

The intensity of 20 μM *Tm*-MreB tryptophan was measured with a Horiba Jobin Yvon fluorimeter. Chemical denaturation was performed with a guanidine hydrochloride solution (6 M Gu-HCl was dissolved in salt-free or high-salt buffer). Guanidine hydrochloride was added to the protein in increasing concentrations in 0.2 M increments. Excitation was set at 295 nm and tryptophan emission was detected at each step between 310 nm and 450 nm at room temperature.

Nucleotide binding and clamping test with TNP-ATP

ATP binding was investigated on *Tm*-MreB with a non-hydrolysing ATP analogue, TNP-ATP. As a result of binding, the TNP-ATP fluorescence intensity increases many times. A fixed concentration (20 μM) of MreB dialyzed in ATP-free buffer was incubated overnight in salt-free (4 mM TRIS-HCl, 0.1 mM $CaCl_2$, pH 7.5) or high-salt (20 mM TRIS-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.5) buffer with 1 μM TNP-ATP, in the absence or presence of 50 μM A22. The next day, the fluorescence intensity of TNP-ATP was measured. Excitation was set at 400 nm and emission was measured between 420 nm and 650 nm using a Jobin Yvon Horiba fluorimeter. The maximum intensity of the spectra (at 540 nm) was used to calculate the binding ratio, and the bound fraction was set to 0 for no binding and 1 for saturation. The following Hill-equation was applied to the curves, where V_{max} is the maximum of the y data series; k is x at half-saturation and n is the Hill coefficient. The binding constant (K_d) of TNP-ATP to *Tm*- MreB was k.

$$y = V_{max} * \frac{x^n}{k^n + x^n}$$

To test ATP competition, 20 μM *Tm*-MreB was incubated with 1 μM TNP-ATP overnight in a salt-free (4 mM TRIS-HCl, 0.1 mM $CaCl_2$, pH 7.5) environment. The next day, non-fluorescent ATP (pH 7.5) was added to the samples and the fluorescence signal of TNP-ATP was measured.

The affinity of ATP to *Tm*-MreB was calculated using the Langmuir single-site binding equation for curve fitting based on previous publications [35].

Steady-state anisotropy

The measurements were performed on a Jobin Yvon Horiba fluorimeter. 1 μM fluorescently labelled vancomycin (Invitrogen) (BODIPY™ FL Vancomycin, FLV) was used to test binding, and the protein concentration was gradually increased. Our test was performed in the presence

of ATP, or polymerising salt, or both. The following equation was used to determine the affinity:

$$y = \frac{y_{min} + ((y_{max} - y_{min}) * \left(((KD + FLV + x) - (((KD + FLV + x)^2) - (4 * FLV * x))^{0,5})\right)}{(2 * FLV)}$$

where y_{min}: the lowest fluorescence anisotropy value, y_{max}: the highest (saturation) fluorescence anisotropy value, FLV: the concentration of fluorescent vancomycin, KD: the binding constant.

Microscopic measurements

Examination of MreB filaments

20 μM *Tm*-MreB protein was polymerised in high salt buffer with or without 100 μM A22. Filaments were labelled with Alexa-488-phalloidin after 2 hours and examined in a Zeiss Elyra SIM S.1 microscope.

Examination of living cells

E. coli cells were grown in LB nutrient solution, shaking overnight at 37 °C. The next day, in the presence of vancomycin, A22, or both, the bacteria were grown in 1 ml of fresh nutrient solution for 1 hour. After that, the cell suspensions were centrifuged (9,400xg, 1 minute), washed twice in PBS (phosphate-buffered saline), and centrifuged again. Finally, the cells were taken up in 1 ml of PBS and then dropped onto an agar pad containing antibiotics for microscopic observations (Olympus IX 81). The length of the cells was determined using the Image J 1.50i program.

The effect of A22 on MreB *in vivo* was investigated using GFP-conjugated MreB in *E. coli*. *E. coli* cells transformed with GFP-MreB plasmid were induced with 2% arabinose in the presence or absence of 50 μg/ml A22. After one hour, the samples were placed on a 1% agarose pad and then examined in a SIM microscope. (Zeiss Elyra S.1 SIM microscope, Szentágothai Research Center, University of Pécs)

Microbiological tests on bacterial populations

Examination of the growth curve and cell parameters

E. coli cell culture was grown in Luria Broth (0.5% yeast extract, 1% NaCl, 1% peptone) nutrient solution at 37 °C in a shaking incubator. The optical density of the cells was detected at 600 nm on a Perkin Elmer Lambda XLS+ spectrophotometer. The length of individual cells was examined using an Olympus IX81 microscope and determined with the ImageJ 1.50i program. For statistics, we ran a one-way ANOVA test, the significance level was determined with the Bonferroni corrected probability test after ANOVA, in the OriginPro 2020 program.

Determination of minimum inhibitory concentration and synergism

E.coli K-12 cell line (Sigma-Aldrich) was used for the measurements. The minimum inhibitory concentration was determined using the microdilution method. On a 96-well plate, 10 different concentration values were set for A22 and vancomycin, with a half dilution, each of them was included 3 times. The obtained results were evaluated after 24 hours of incubation at 37 °C. The synergistic effect was determined using a microdilution checkerboard assay. The optical density of the bacterial suspension was set to 0.12 (OD_{600}, McFarland standard 0.5), and a 500-fold dilution with LB nutrient solution was used to obtain a concentration of 2*105 cells/ml. 50 µl of 2-fold serially diluted vancomycin was added horizontally to the 96-well plate, and another 50 µl of serially diluted A22 was added vertically to the wells. 100 µl of bacterial culture was added to each well, so the cell culture and antimicrobial agents were finally diluted to half (this was taken into account when measuring the dilution series). The first column contained only nutrient solution as blind, and the second contained only *E. coli*, diluted to half in LB nutrient solution as a control. After 24 hours of incubation at 37 °C, the results were measured with a microplate spectrophotometer (Thermo Electron Corporation, Multiskan EX), the optical density was determined at 535 nm. Using the smallest MIC value of the antimicrobial agents, the value of the fractional inhibitory concentration index, i.e. ΣFICI (fractional inhibitory concentration index), was calculated using the formula below. If ΣFICI≤0.5, we can talk about synergism, so the two antibacterial agents support each other's effect.

$$\Sigma FICI = \frac{FICI_{A,together}}{FICI_{A,itself}} + \frac{FICI_{B,together}}{FICI_{B,itself}}$$

RESULTS AND DISCUSSION

During the preparation of *Thermotoga maritima* MreB, we used a new method that allows the production of the native conformation of the MreB protein and results in a large amount of soluble, pure protein. We worked in *E. coli* Bl21 ArcticExpress (DE3) system. This cell line is advantageous for native exploration, as expression takes place at a low temperature (10-13 °C), so the chance of formation of inclusion bodies, i.e. protein aggregates, is lower than in other previously used BL21 cell types (DE3; DE3 pLysS, Rosetta). It is worth highlighting that the system contains endogenously expressed chaperone proteins, which ensures the normal folding of the protein, i.e. promotes the formation of the native conformation during expression.

The reason for the different thermal stability measured in saline and salt-free buffer environments may be the formation of a salt bridge within the molecule. It is an earlier observation that the salt bridge formed between oppositely charged amino acids can stabilize or even destabilize the protein structure [36]. In the case of *Thermotoga maritima*, a salt bridge forms between amino acids K49 and E204 [10]. Since one of these amino acids is located on the D-loop of *Tm*-MreB, it may play an important role in the conformational change occurring during polymerisation. In our opinion, during the cell lysis and initial purification steps, NaCl has a stabilizing role in the *Tm*-MreB structure, however, it prevents the formation of the salt bridge. By removing NaCl, the formation of salt bridges becomes possible, which increases structural stability, makes the protein soluble, and increases thermal stability. This salt bridge-induced increase in thermal stability has also been described for other temperature-tolerant proteins [17]. On the other hand, MreB presumably has other binding partners that stabilize the protein within the cytoplasm.

During the thermal denaturation of *Tm*-MreB, no spectral shift in the tryptophan fluorescence emission wavelength towards red wavelengths can be observed in the case of actin. This can be explained by the fact that the single tryptophan amino acid of *Tm*-MreB is located in a different place in the molecule than the four tryptophans of actin, it is more exposed to the solvent. For this reason, in the case of *Thermotoga maritima* MreB the conformational change induced by heat denaturation does not cause a spectral shift, only a decrease in fluorescence intensity.

It is an interesting observation that during chemical denaturation, the local environment of the same protein and the same amino acid changes in a different way than due to heat. In this case, the red shift was observed. From this, we can conclude that chemical denaturation and thermal denaturation cause a different conformational change in the *Tm*-MreB protein. As a

result of the chemical treatment, the environment of the tryptophan of the protein changes to a greater extent.

It was previously described that the four tryptophans of eukaryotic actin contribute differently to the intrinsic fluorescence intensity of actin [37]. The tryptophan amino acid Trp- 79 of actin is the most accessible to the solvent compared to the other three tryptophan amino acids of actin. The position of the single tryptophan of *Tm*-MreB is located separately from the tryptophan amino acids of actin.

The non-hydrolysing ATP analogue, TNP-ATP, proved to be suitable for describing the nucleotide binding of the *Tm*-MreB. In support of our thermal denaturation studies, where we found a more stable conformation of *Tm*-MreB in the salt-free environment, we also found that in salt-free conditions *Tm*-MreB binds TNP-ATP more strongly than in the presence of high concentrations of monovalent ions. On the other hand, we found that the MreB-specific inhibitor A22 does not inhibit the binding of TNP-ATP to MreB under any conditions. Finally, we concluded that ATP and TNP-ATP compete with each other. The 2 μM affinity of ATP to *Tm*-MreB means a relatively weak binding, it can be assumed that other factors or binding partners in the cytoplasm can improve this.

Our microscopic studies showed that in the presence of A22 larger elements than monomers are formed, the protein may aggregate or precipitate. These structures cannot be called filaments, as in the case of protein fibres formed in the presence of a polymerising salt.

Examining the effect of A22 on polymerisation, we can state that using a low concentration of A22 accelerates and promotes protein assembly, but above 50 μM, its inhibitory effect prevails and slows polymerisation. It prevents the breakdown of inorganic phosphate after the hydrolysis of ATP. In our opinion, A22 can maintain an ADP-bound state on MreB, thus the protein cannot perform its cell shape maintenance function inside the cell. The protein therefore begins to assemble and form oligomers, as confirmed by microscopic and sedimentation tests. However, this is not the correct, native state of MreB in the cell. As a result, the bacterial cells become rounded, lose their rod shape, and ultimately lyse and die.

Regarding vancomycin, we found that fluorescent vancomycin can bind to the protein *in vitro*. Fluorescent vancomycin was previously only used on living cells. We described the affinity of BODIPY-vancomycin to *Tm*-MreB under different conditions. These binding rates (4-8 μM) can also be said to be weak, as in the case of TNP-ATP. Further studies are in progress to determine whether this binding may be specific. Similar to A22, vancomycin affects the polymerisation of MreB in two phases: it accelerates it below the minimum inhibitory concentration and slows above it.

When the two antimicrobial agents were used together, the growth of *E. coli* bacteria was inhibited more than the two compound was used separately. As a result of treatment with vancomycin, the cells became slightly shorter compared to the control, however, as a result of A22 treatment the cells became rounded and shortened significantly. When the two compounds were used together, the effect of A22 was more prominent, and we found cell sizes similar in magnitude to the A22 treatment. From these results, we concluded that there may be a synergistic effect between vancomycin and A22, i.e. they can strengthen each other's antimicrobial effect. And the checkerboard essay showed a very strong synergism between the two compounds. A22 has an antimicrobial effect only on Gram-negative bacteria, the MIC value on Gram-positive bacteria is very high or cannot be determined at all. In contrast, vancomycin is an antibiotic that acts on Gram-positive bacteria. In our experiments, however, vancomycin managed to exert a significant effect on Gram-negative *E. coli*. Presumably, as a result of the A22 treatment, the cell wall of the Gram-negative bacteria is weakened by inhibiting the function of the MreB protein, so that the antibiotic acting on the Gram-positive bacteria can also enter the cell. This can therefore be a new way to prevent bacterial resistance, to sensitize bacteria to existing antibiotics.

I summarize the results of our work as follows:

- We managed to develop a purification protocol that resulted in a large amount of pure and soluble native conformation *Tm*-MreB protein, which proved to be functional.
- We determined the heat denaturation values of the protein in high salt and salt-free environments, in the presence or without ATP.
- We found that *Tm*-MreB is more stable in the presence of ATP and in salt-free buffer. However, due to the removal of imidazole, it is necessary to include a purification step in a high-salt buffer.
- The salt bridge formed between amino acids K49 and E204 stabilizes *Tm*-MreB. During chemical denaturation, we observed a shift of the tryptophan emission maximum of MreB to a longer wavelength range. This suggests that during chemical denaturation, the environment of tryptophan becomes more polar, presumably more accessible and exposed to water.
- We did not observe this phenomenon in case of heat denaturation, from which we conclude that the conformational change of *Tm*-MreB is different during chemical and heat denaturation.

- We determined the binding affinity of TNP-ATP and ATP to *Tm*-MreB, which was 3.7 and 2 μM, respectively. These fit well with those previously described for other MreB proteins [25, 26]. We found that TNP-ATP is bound by *Tm*-MreB with higher affinity in a salt-free environment.
- According to our studies based on light scattering, nucleotides do not affect the rate of polymerisation.
- A22 does not affect nucleotide binding or TNP-ATP binding. At low concentrations, it accelerates protein polymerisation, at high concentrations it inhibits it. Prevents the breakdown of inorganic phosphate.
- In addition of polymerising salt A22 creates oligomers or aggregates, which is yet to be clarified. However, it is sure to interfere with the natural assembly process of MreB. Based on our experiments, we believe that A22 creates an ADP-bound state, which prevents MreB from performing its shape-maintaining function in cells.
- Another finding of our work is that fluorescent vancomycin can bind to MreB, the binding constants were also determined in different salt and nucleotide environments. Vancomycin binds to MreB with an affinity of 8.329 ± 0.571 μM, in the case of ATP bound MreB the binding constant was 6.894 ± 0.509 μM. The vancomycin binding constant was 5.59 ± 1.21 μM in the presence of polymerising salt, and 4.048 ± 0.723 μM in the presence of ATP and salt. Like A22, it affects the polymerisation of MreB in a biphasic manner.
- Vancomycin inhibited the growth of *E. coli* bacteria better than A22, but the inhibitory effect was stronger when the two antibiotics were used together. Both compounds had an effect on the length of the cells, however, in the presence of A22, the cells became significantly shorter and rounder.
- We proved with microbiological assays that there is a synergistic effect between the two antimicrobial compounds and quantified with the FICI values. Using different combinations of the two antimicrobial agents, the following concentration values were obtained: 0.5 μg/ml; 0.375 μg/ml; 0.31 μg/ml; 0.25μg/ml and 0.1875μg/ml. Based on these, we can conclude a strong synergistic effect, the antimicrobial agents amplify each other's effect.

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