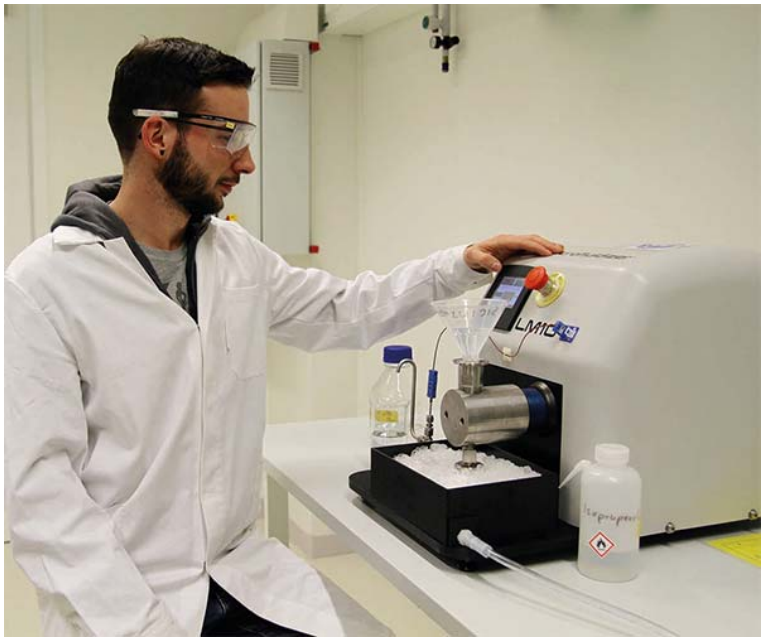
More insight into how proteins work by modulating structures

Researchers from the Organic Chemistry section of VU University Amsterdam are gaining more and more influence on how they work from fundamental knowledge of 3D structures of proteins. Among other things, they build in peptides that are synthesized from non-natural amino acids. Sven Hennig, who has been working at Tom Grossmann's group since 2017, has set up a crystallography X-ray facility for structural analysis. An essential purification step - the release of the proteins from an E. coli expression system - is done with a microfluidizer.



PhD student Sebastian Kiehstaller uses the LM10 microfluidizer from Microfluidics, supplied by Salm and Kipp, for the first step in the protein purification process expressed in E. coli. With this, the proteins can be reliably and quickly released from the cell suspension.

How do the tertiary and / or quaternary structure of proteins change under the influence of interactions they enter into, and how can you control or optimize those interactions by modifying the protein structure? That is a nutshell where researchers from the Grossmann group, which is part of the Organic Chemistry section of the VU, try to get a grasp of a wide range of proteins, which are often involved in transcription and regulation processes. Assistant professor Sven Hennig (who forms the research group together with Tom Grossmann and five PhDs, two post-docs and four master students) explains how such research projects work.

“We always start from the crystal structure or an NMR plate so that we know exactly the position of every atom in the structure. The effect of a protein is generally based on the interaction with a 'binding partner', which takes place in a certain part of the protein. By simulating and modulating these binding partners, we can better understand the binding mechanism. With that knowledge you can then make targeted modifications to the protein in order to optimize or inhibit a certain effect. Or imitate the binding partner in such a way that it does better than the natural form. In such projects, we primarily acquire fundamental knowledge, but it soon comes into contact with

applications. Take for example a tumor protein. If you know the binding partners of that protein, can modulate your structures in such a way that the interaction no longer leads to an oncogene effect. In this way our work can form the fundamental basis for the search for drugs that specifically target that protein. "However, clinical application is never just the goal. "We are particularly interested in understanding the interaction in order to come up with new ideas from there. We are not so much looking for molecules that can be used for something, but want to acquire fundamental knowledge that can also be used for other protein interactions and modulations.

The LM10 microfluidizer from Microfluidics presses a cell suspension through a shearing cell, a ceramic unit, which becomes increasingly narrow and destroys the cell at a given moment. Then the lysate, with the proteins in it, goes through a part that cools very efficiently.

Peptide synthesis

An important part of the work is in the synthesis of peptides. These are primarily intended to mimic the pieces of protein that are important for the interaction. It is not always easy to get binding partners in the right place to modulate protein-protein interactions. Using a piece of approximately ten amino acids representative of the interaction makes it a lot easier. And also modulating (changing the structure of the peptide to find a better binding partner or modulator for the interaction in question) thereof is relatively simple with the help of chemical synthesis. Where many people think of a sequence of some of the 22 natural amino acids occurring in the cell in peptides, this is only the starting point for their VU researchers in their synthesis work. "To be able to understand things better, we need to be able to apply more diversification to the peptides. For this we use, for example, peptides that do not occur in nature, or peptides that can bind with themselves, for example by closing ring structures. We can synthesize these building blocks ourselves in the lab, but because this branch of sport has grown considerably, you can increasingly buy them simply, "says Sven Hennig. The peptidomimetics are a separate category in that respect, in which all

structural properties of a 'wild type' peptide are missing. This de-peptidizing to a total 'mimick' of the peptide goes so far that it no longer even contains the original backbone of the peptide. In fact, it is then a 'small molecule' that mimics a peptide. you can also buy them more often, "says Sven Hennig.

Assistant professor Sven Hennig, who has been working at Tom Grossmann's group since 2017, has set up a crystallography X-ray facility for the structural analysis of proteins.

Stabilize

In addition to functionality, the stability of protein structures also plays an important role in research. "For example, cysteines in the protein chain naturally provide extra stability by entering into sulfur bridges. However, if you want to incorporate non-natural amino acids into the protein, then that is not all that straightforward: bacterial systems are not as easy to incite to pick up non-natural amino acids and put them in the protein. In our group, we therefore started looking for other ways to stabilize the proteins. We focused on stabilizing the tertiary structure through the use of larger cross-linkers. In this 'in situ cyclization of proteins' (INCYPRO) approach we have built in modular crosslinks in native proteins, which consist entirely of proteogenic amino acids, that is, the amino acids that are incorporated directly into the peptide chain during the translation of the genetic code into a polypeptide. We have succeeded in linking a chemical linker to the cysteine residues in the protein in three places. After reaction with a tris-electrophile, a cyclization is formed in situ. This provides more stability, which we have recently demonstrated for, among other things, a sortase A, an enzyme used in protein engineering for in vitro post-translational modification of proteins. So we can now use standard molecular-biological techniques to modify genes that code for a protein with easily approachable cysteines, "explains Sven Hennig. an enzyme used in protein engineering for in vitro post-translational modification of proteins. So we can now use standard molecular-biological techniques to modify genes that code for a protein with easily approachable cysteines,

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Due to the greater stability of the structure, this enzyme can also carry out a certain reaction at higher temperatures and is more resistant to detergents such as urea. Such results are not only therapeutically interesting, because the protein is better able to deal with stress factors, but also from a biotechnological perspective. "Certain enzymes cannot now be used in large installations because, for example, the pressure or temperature is too high, or because certain organic solvents are being used. We have now developed the tools to further stabilize such proteins, to make them more robust, so that they can be used on a large scale in biotechnological processes. In that regard, the law 'if we loose structure, we loose activity' also applies here. The reason for a lower biological activity may be that the structure is not satisfactory, is unstable. If we stabilize the structure, we can do better, as we can see from the melting curve, for example. "

Pure protein

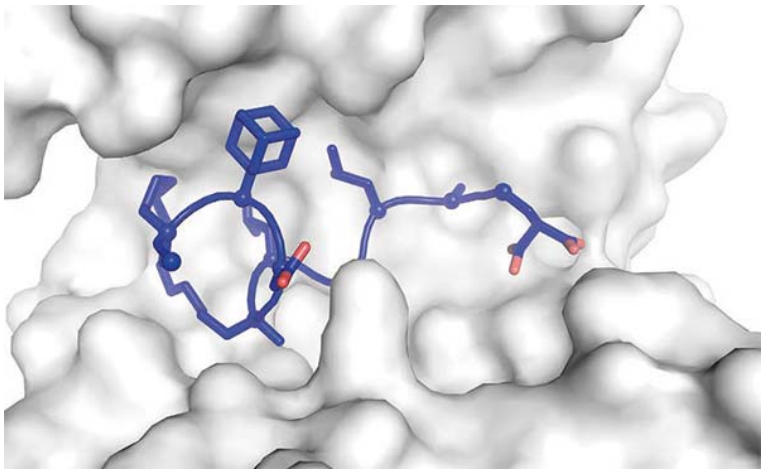
The 'proof of the pudding' in making all those new structures is that you can also make beautiful 3D pictures of them. For this, Sven Hennig, soon after joining Tom Grossman's group in 2017, set up an X-ray facility with associated protein purification in the department that is specifically aimed at crystallography of proteins and carbon-based small molecules. A facility that is not only intended for the Organic Chemistry group, but can also be used for fellow researchers within the AIMMS (Amsterdam Institute for Molecules, Medicines and Systems) located in the O2 building. One of the researchers who works a lot with the X-ray facility is PhD student Sebastian Kiehstaller,

"Biochemists are used to cleaning everything well.

Just like with cooking: someone has to do the dishes! "

"The practical experience we gained in Dortmund with crystallography of proteins came in handy for us at VU University Amsterdam when setting up the X-ray facility and - not unimportantly - the associated protein purification. This is because for most projects it is necessary to purify proteins that are expressed in E. coli. The first step is to get the protein out of the cells. For this we use the LM10 microfluidizer from Microfluidics, which is supplied by Salm and Kipp. This type of device, which we also used to full satisfaction in Dortmund, presses a cell suspension through a shearing cell, a ceramic unit, which becomes increasingly narrow and destroys the cell at some point. Then the lysate with the proteins in it goes through a part that cools very efficiently, which is essential. This is because a lot of heat is generated in this process, which takes place under high pressure, and proteins cannot withstand that well. We have chosen this method over sonication, which was

initially applied to this group. This involved too large a batch-to-batch variation for our experiments and was therefore not efficient enough. The microfluidizer is a very robust device, reliable and fast and easy to use. Cleaning is also not a problem: just use water (or sometimes an isopropanol mixture) instead of the cell suspension, "says Sebastian Kiehstaller.



X-ray structure (pdb-id: 5 µm4) of a macrocyclic peptide (blue) with two non-natural amino acids and a crosslinker that binds to its target protein (white, 14-3-3).

Source: Structure-Based Design or Non-natural Macrocyclic Peptides That Inhibit Protein-Protein Interactions. Kruger, DM, Glas, A., Bier, D., Pospiech, N., Wallraven, K., Dietrich, L., Ottmann, C., Koch, O., Hennig, S., Grossmann, TN (2017) J. Med. Chem. 60: 8982-8988.

Beautiful protein crystals

After further purification by centrifugation (to get rid of insoluble membrane fragments and cell residues, among other things) and, depending on how difficult the separation is, one or more steps with affinity chromatography or SEC, you have the pure protein in a solution. Then you are not there yet, because for X-ray crystallography you have to have a semi-solid state of the protein, so you still have to get rid of most of your solution. According to Sven Hennig, that part is perhaps even more difficult than the X-ray process itself. "It takes a lot of time to come up with good crystals. For this we use a technique that is based on vapor diffusion. For this we distribute the protein on a 96-well plate, each well being filled with a differently composed buffer. With these plates with pre-made screening conditions, that you can order ready-made, you can very efficiently arrive at the best conditions for the protein in question via trial-and-error. By sealing the wells airtight and applying a vacuum, you start the process of vapor diffusion from the droplet, which is initially 200 nl large, in the reservoir on the bottom of the well. This way you increase the concentration of the protein and force the protein to precipitate. In the optimum case, a crystal will be formed and you can get started with the X-ray and dissolve the structure. that in the first instance is 200 nl in size, operates in the reservoir at the bottom of the well.

If you have optimized the conditions for a particular protein, then you can also work with larger amounts of protein. We try to make as much protein as possible in a run, because growing cells takes a lot of time. The microfluidizer is also of value in that respect: it makes absolutely no difference whether it has to process much or little cell suspension. You turn it on and it just does its job!"