D2AM-Bio: Steps towards Better Protection of Tobacco Crops and New Design of Antibiotics

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Many protein structures were solved in 1995 and 1996 with data collected on D2AM, the CRG beamline on the bending magnet BM2. A new cytochrome structure, mutants of a hydrogenase, β-lactamase, structures of molecular complexes, such as protein-inhibitor (psychrophylic amylase) or protein-cofactor (17β HSD) complexes were determined using essentially molecular replacement. Three structures were solved ab initio with multiwavelength anomalous diffraction (MAD) data collected at the Pt, Se or Fe absorption edge. The first two, *cryptogein and MurD*, will be presented below; the refinement of the third one, a new form of cytochrome c3, is currently in progress at 1.7 Å resolution. The beamline was also successfully used for mosaicity measurements on protein crystals grown in microgravity, the angular resolution of these measurements being of the order of 3.

These results demonstrate the great flexibility of use of the D2AM beamline for protein crystallography. Instrument and software allow one to collect easily diffraction data at n wavelengths, where n can be 1, 2, 3 ... or up to 20 values, at any temperature between -180°C and room temperature. In addition, these results also demonstrate the good quality of data obtained with the XRII-CCD detector and evaluated with XDS [1]. As an example, a lysozyme data set collected at 2Å, the diffraction limit of the crystal, showed an R_{sym} of 2.5%.

An Original 3-D Protein Structure

Fungi of the genus Phytophthora are a major natural cause of crop devastation. For example, they are responsible for the blight of potatoe plants (*Phytophthora infestans*) which destroyed the potato crop in Ireland in 1845 causing the great starvation which is at the origin of the massive emigration of Irish to the United States, and the mildew of vineyards (*P. vignae*) which destroyed the French vineyard at the beginning of the century, or the black shank of tobacco plants (*P. parasitica* var. *nicotianae*). Even nowadays one third of the world tobacco production is damaged by this infection. The baleful action of the fungus is due to the invasion of the roots and stalks of the plant by the mycelium of the fungus producing the lethal perishment (Figure 1). However most species of the *Phytophthora* fungi family do not have such a catastrophic effect on the plant they infect, but they induce only localised necrosis of leafs. In effect most of these fungi secrete a small molecule called *elicitin* and plants have developed a system of defence against fungal invasion, which is triggered off by this molecule. The localised necrosis of the leaf can be considered as a manifestation of this defensive response. One may think for instance that the plant destroys the locally infected tissue. The virulence of the few fungi, which induce a lethal pathogenicity, like *P. parasitica* on tobacco, is due to the absence of elicitin by these fungi. They have the

Fig. 1: a tobacco plant infested by the fungus Phytophhtora parasitica nicotianae. Cryptogein, a protein secreted by Phytophhtora cryptogea, a fungus belonging to the same family, induces an immunisation of tobacco plants against P.p.nicotianae. The toxicity of the protein produces nevertheless a non lethal localised necrosis of the plant.

particularity of not having elicitin secretion. The reactivity of plants to elicitin is not very specific. Once the plant

has acquired this mechanism of defence by contact with an elicitin, it is protected against any further fungal invasion, even by a fungus of *Phytophthora* family which do not have elicitin. This was demonstrated by injecting elicitin to tobacco plants and testing afterwards their resistance to infection by *P. parasitica*. The analysis of the properties of the elicitins shows first that they belong to two classes, highly necrogenic elicitins and less necrogenic elicitins, the later being 50-100 times less necrogenic than the former, and secondly that necrogenicity and ability to activate the plant defence mechanisms are not correlated. This evidence allows one to imagine new approaches for agricultural fungicid developments based on elicitins. It might be possible to engineer an elicitin, non-necrogenic but powerful for protecting plants. To achieve this end, it is necessary to know first in great details the structure of the molecule and the characteristics of this structure which explain its properties.

All members of the elicitin family identified up to now have very high sequence homology. They constitute a *new family of proteins*. No primary sequence homology with any other known protein was found. The comparison of the sequence of the highly necrogenic elicitins and the less necrogenic ones has allowed to select several potential necrotic activity-determining residues. But it seems that the residue at position 13 plays a key role in this property. It is a lysine in the highly necrotic elicitins and a valine in the less necrotic elicitins. This was demonstrated by replacing Lys13 by a valine by site-directed mutagenesis. The mutation produced a dramatic alteration of the necrogenicity of the protein. In order to identify more structural features important for the necrotic activity of elicitins, the crystallographic structure determination of cryptogein (CRY), secreted by *Phytophthora cryptogea*, was undertaken.

In collaboration with the Laboratory of Dr. Pernollet (INRA, Jouy-en-Josas, France), CRY was crystallised in high salt concentration (4.9 M NaCl) in space group $P4_122$ (a = b = 47 Å, c = 137 Å). The absence of sequence homology with structurally characterised proteins directed efforts towards the isomorphous replacement method. The extensive search for heavy-atom derivatives yielded only one poorly phasing tetrachloroplatinate derivative, with a rather low atomic occupancy. This difficulty might be related to the unusual property of the molecule which lacks for several amino-acids which are important for heavy atom binding like His, Glu, Arg. The six cysteines found in the sequence are involved in disulfide bridges. The crystal structure was therefore solved by the multiwavelength anomalous diffraction phasing method. Four data sets were collected in the vicinity of the platinum LIII absorption edge ($\lambda \approx$ 1,0723Å). A native data set was collected in addition for completion. In the first stage, the MAD platinum derivative data were phased using the programs MLPHARE [2] or HEAVYv4 [3]. The resulting electron density map was interpretable but its overall quality was not good enough for a clear identification of all secondary structural elements, even after solvent flattening by the program DM [2]. In contrast, the maximum-likelihood heavy-atoms parameters refinement performed using the program SHARP [4] coupled to the addition of the native data set in the phases calculation and the use of the solvent flattening program SOLOMON [2] were decisive in obtaining a clear, unambiguous electron density map into which the molecular model could be constructed readily. The atomic model, refined with the program X-PLOR [5], shows a final R-factor of 21,8 % (R_{free} = 27,9 %) between 7 and 2.2Å.

The structure of CRY is made of six α-helices and one antiparallel β sheet (Figure 2) [6]. It characterises a *new type of protein folding*. The necrogenicity-related residue 13 is located on the surface of the molecule, well exposed to the solvent on a rather peripheral site. Why this single residue has such an effect on the necrogenicity is not known. Two interesting remarks can be made. First, an original structural feature is observed, consisting of an omega loop facing the antiparallel β sheet. This part of the molecule, which has been called *beak-like motif*, corresponds to a highly conserved region of the sequence and therefore it is likely to have an important functional role. It could be the recognition site with a partner. Secondly, the side chain of Tyr87 is plunging into the hydrophobic core of the molecule. In the crystal structure of a mutant of CRY, the side chain of Tyr87 was found oriented outside of the molecule, creating a hydrophobic cavity in which the electron density of a ligand has been identified. What kind of a ligand is it ? Very little is known presently about the function of this molecule by the fungi and how the protein interacts with plants. Further functional analysis of the molecule will require more information about these key questions.

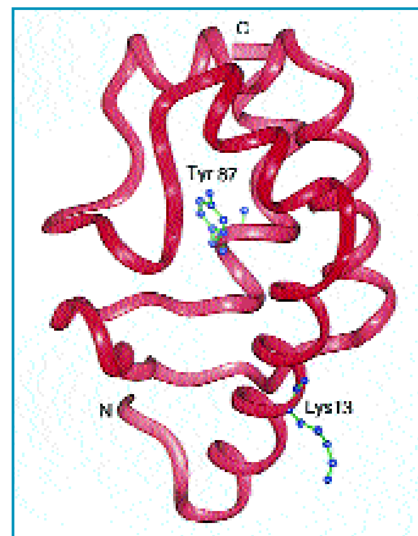

Fig. 2: Folding diagram of cryptogein showing the original beak-like motif on the left side of the structure, the hydrophobic cavity near Tyr87 and the location of the residual 13, critical for the toxicity of the molecule.

SEARCHING FOR NEW ANTIBIOTICS

Antibiotics have been in extensive clinical use over the past 50 years in antibacterial therapies, saving countless lives by killing the bacteria responsible for infectious diseases such as pneumonia, meningitis, and tuberculosis. As a result of the high efficacity of antibiotics, such as the β-lactams (penicillins and cephalosporins) and the glycopeptides (vancomycin), the general population now expects that any bacterial infection will be easily cured by one of these miracle drugs. However, the days of the miracle drugs may be coming to an end as their therapeutic value is now largely reduced or totally annihilated by the emergence and spreading of various resistance mechanisms (for reviews see Science 264: 360-393, 1994).

The existence of antibiotic-resistant strains of bacteria is nothing new, penicillin resistance strains of bacteria were observed only a few years after its clinical debut in 1942. Nonetheless, for decades the pharmaceutical industry has managed to stay ahead of the resistance by slightly modifying the structures of their antibiotics. But the modifications have continuously been proven to be only a temporary fix for the problem as the bacteria quickly adapt to the modifications. Clearly, what is needed is not a slight modification of the classical antibiotic but rather a change in the target.

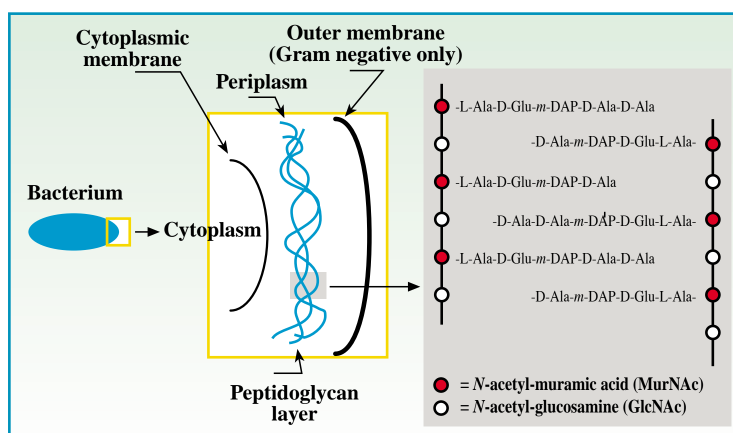

Fig. 3. Structure of the bacterial peptidoglycan layer. The bacterial cell wall is constituted by the assembly of parallel long peptidoglycan chains which are crosslinked by bonds established between the side chains, forming a huge 2-dimensional bag shaped single macromolecule. The bacteria cannot live without this cell wall. Many antibiotics act as inhibitors of the peptidoglycan synthesis.

The majority of the antibiotics in clinical use inhibit the biosynthesis of the peptidoglycan layer, a large mesh-like structure which completely surrounds the bacterial cell. The peptidoglycan (Figure 3) is formed by linear repeating disaccharide chains which are interconnected by short polypeptides which are derived from the pentapeptide unit: L-Ala-D-Glu-*m*-Dap-D-Ala-D-Ala. This assembly is essential to the survival of the bacteria in that it prevents the bacteria from bursting from the high internal osmotic pressure. The antibiotic penicillin is an inhibitor of the enzymes which cross-link the peptide chains of the peptidoglycan, one of the later steps of the peptidoglycan synthesis. The new approach, followed in the framework of the present study, is to look for inhibitors of an enzyme involved in the earlier stages of peptidoglycan biosynthesis. As such, the enzymes of the biosynthetic pathway of the UDP-*N*-acetylmuramoyl-pentapeptide, the peptidoglycan precursor, represent attractive targets for the development of new antibiotics.

In the search for new antibiotics detailed structural knowledge of the target enzyme is of primary importance. Using this knowledge, strategies can be developed for the rational design of novel inhibitors. The present structure determination concerns the enzyme uridine diphosphate N-acetylmuramoyl- L - alanine: D -glutamate ligase (MurD). The work stems from a collaboration between the Laboratoire de Biochimie Moléculaire et Cellulaire (Université de Paris-Sud, Orsay) and the Laboratoire de Cristallographie Macromoléculaire (I.B.S., Grenoble). MurD catalyses the addition of D-glutamate to the nucleotide precursor UDP-MurNAc-L-Ala forming a L-Ala-D-Glu linkage that is present in the peptidoglycan of all eubacteria. Inhibition of MurD stops the biosynthesis of the precursor and therefore interrupts the construction of the bacterial cell wall.

The structure of MurD was solved to 2.8 Å by MAD analysis of the selenomethionyl protein using data collected from a single frozen crystal on the D2AM beamline [7]. As much as 1,120 CDD images of 1242 x 1152 pixels were recorded and subsequently treated by the program XDS [1]. Approximately 61,000 reflections were collected at each of the four wavelengths (remote: 0.9827 Å, f" inflection: 0.9797 Å, f" peak: 0.9794 Å, remote: 0.9762 Å) giving values of R_{sym} between 3.6 and 4.4 %. The seleno-MurD (437 a.a., 47 kDa) was obtained by expression in Met - *E. Coli* and contained 12 Se atoms. The values of f' and f" of Se near the absorption edge were determined in the usual way by reference to the fluorescence spectrum of the protein crystal measured on the diffractometer. The Se partial structure factors, calculated using MADSYS [8], were used with the direct methods option of SHELXS-86 [9] to locate the selenium sites. Refinement of the heavy atom parameters with calculation of the phase probabilities was performed with MLPHARE [2]. The elapsed time between the crystallisation of the seleno-MurD (Feb. 96), the MAD data collection on the D2AM (late Feb. 96) and the resolution of the structure by obtaining the first interpretable electron density map (late Apr. 96) was less than three months. The calculated phases were later improved by including mercury and iodine derivative data of the seleno-MurD collected using an «in-house» X-ray source.

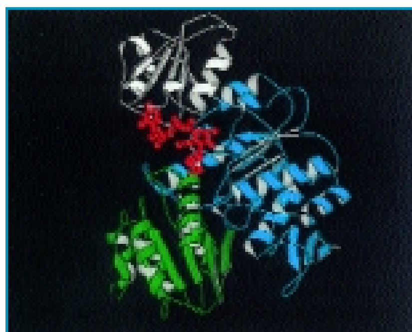

Fig. 4. Ribbon diagram of the binary complex of MurD and UMA. Domain 1 is shown in pink, domain 2 in blue, domain 3 in green and UMA in red.

The MurD structure is currently refined to 1.9 Å resolution using data collected from a crystal of the native enzyme/substrate complex on the D2AM beamline. It is the first structure reported for a member of the Mur ligase family. The structure comprises three domains of topology each reminiscent of nucleotide-binding folds: the N- and C-terminal domains are consistent with the dinucleotide-binding fold and the central domain with the mono-nucleotide-binding fold (Figure 4). The substrate UDP-N-acetylmuramoyl-L-alanine (UMA) is bound in the cleft formed between the N-terminal and central domains. Structural analysis supports a mechanism which proceeds by phosphorylation of the C-terminal carboxylate of UMA by the γ-phosphate of ATP to form an acyl phosphate intermediate, followed by nucleophilic attack by the amide group of D-glutamate to produce UDP-N-acetyl-muramoyl-L-alanine-D-glutamate, ADP, and inorganic phosphate. ■

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