STUDENTSKÁ PŘEHLÍDKA

S1

X-RAY AND NMR STUDY OF PROTONATION STATE OF PHARMACEUTICAL AMIDES

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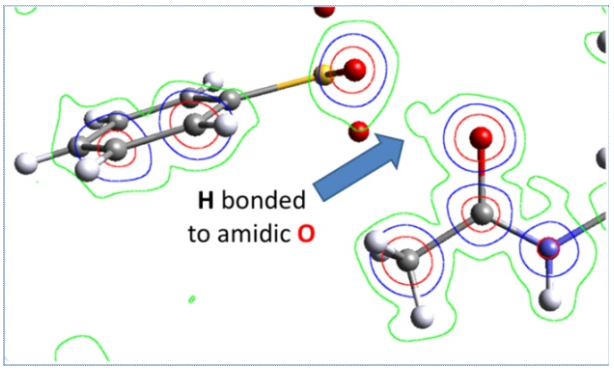
The search for new solid forms of an active pharmaceutical ingredient (API) is an important step in a drug development. Salts and co-crystal are multicomponent solids but in different protonation (ionization) states. In salts, there is a proton transfer between the molecular components, making it contain cations and anions. On the other hand, co-crystals are made up from neutral molecules held together by non-bonded interactions. Often, an API has a low water solubility, which then leads to a low oral bioavailability. This physico-chemical problem can be solved by a co-crystal or salt formation. An API with one such problem is agomelatine, a melatonergic antidepressant. However, agomelatine is an amidic compound and, since amides are generally considered as neutral (non-ionisable), it was quite a surprise, when agomelatine, in the combination with certain acids, produced salts. Several co-crystals were prepared as well. The novel crystalline phases were prepared either by a slow evaporation or precipitation of the solution of agomelatine with the respective acid. The prepared solid phases were analyzed by powder X-ray diffraction and the structures were solved from single-crystal or powder X-ray diffraction data. The crystal structures of six agomelatine salts (with hydrobromic, hydroiodic, triiodic, sulfuric, methansulfonic and benzensulfonic acids) and of five co-crystals (with citric acid, maleic acid, oxalic acid, 4-hydroxybenzoic acid and hydroquinone) were solved. In addition to the anhydrous forms, in the case of three of the salts (with hydroiodic, sulfuric and methansulfonic acids), the structures of hydrated/solvated forms were described as well. In all the salts, the agomelatine molecule was positively charged. Specifically, the amide oxygen was protonated. The proton transfer and the salt formation were also confirmed by solid state NMR and the pK_A calculation. The comparison between single-crystal X-ray diffraction, solid state NMR and pK_A data revealed a strong correlation, meaning that all three methods can be reliably used to distinguish between salts and co-crystals. For pharmaceuticals, the determination whether the material is a salt or a co-crystal is interesting not only academically, but also from the regulatory point of view. Therefore, our findings may play a crucial role in the future development of the multicomponent solid phases of agomelatine and other amidic pharmaceutical compounds.



A slant Fourier map of the observed electron density and the molecular model of agomelatine – benzensulfonic acid (a proton transfer in a salt).

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S2

PHARMACEUTICAL COCRYSTALS AND THEIR APPLICATION

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When speaking of multicomponent solid forms of organic molecules, terms such as solvates, hydrates, salts and cocrystals are often used. From these forms, the cocrystals have recently gained much attention. By the term cocrystal, a stoichiometric multicomponent solid with host and guest molecules arranged in a common crystal lattice, is meant. In the case of pharmaceutical cocrystal, one of the components is an active pharmaceutical ingredient (API); the other is a pharmaceutically acceptable coformer. In the generic pharmaceutical industry, cocrystals are used to widen the portfolio of solid forms of APIs, from which the form with the optimal physico-chemical properties is formulated into the drug product.

To obtain as many solid forms of API as possible, a systematic screening is undertaken during the early development stages of the compound. Thus, polymorphs and solvates are obtained from various solvent-based techniques. When combining API to counterions or coformers (in many cases the counterions and coformers are the same compounds), a salt or a cocrystal can be prepared. Such forms can be further screened for polymorphs and solvates/hydrates.

The uniqueness of the novel crystalline form is usually determined by X-ray crystallography. In the past, the powder X-ray diffraction patterns were used to differentiate between the forms of pharmaceutical substances (especially in patent literature); more recently, the structure determination from single crystal X-ray diffraction is the most common technique to describe the novel form.

When deciding between a cocrystal and a salt, the difference often lies merely in the position of a proton between its donor and acceptor. To determine the position of a proton and, thus, decide whether the new form is a cocrystal or a salt, can be achieved via various analytical techniques; solid state NMR, infrared and Raman spectroscopy being useful tools as well as neutron diffraction. The decision is crucial for registration. While the pharmaceutical salts of API are not considered equivalent with API, the cocrystals of API are considered as its intermediates, thus permitting easier acceptance by authorities. As such, they can compete with polymorphs and hydrates of API.

In the presented case studies, two cocrystals of pharmaceutical substances are described.

Cocrystal with fumaric acid (2:1) is a stable form of a prodrug. However, in the marketed drug product, a mixture of salt fumarate (1:1) and the cocrystal was identified, as both forms are related and convert from one to another. The challenge of stabilizing the salt, thus avoiding the conversion into the cocrystal, has been faced. The excipients and wet granulation contribute to the conversion to the cocrystal, causing physical impurities in the bulk. The pH measurements of excipients mixtures have shown that increased pH accelerates the conversion. The addition of citric acid to adjust pH has proven successful in stabilizing the desired form during the formulation.

In the other case, a pharmaceutical salt used for the treatment of angina pectoris was screened for cocrystals, and a hit with (*S*)-mandelic acid was identified. Upon structure determination and characterization by solid state analytical techniques, the formation of cocrystal was confirmed. Its physico-chemical properties were compared with other solid forms of the API by stress studies. Both physical and chemical stability was satisfying, selecting the cocrystal as a drug form comparable with the form used in the original drug product.

While the cocrystal with fumaric acid was an undesired phase in the developed drug product, the cocrystal with (*S*)-mandelic acid was chosen as the form with optimal properties for formulation. The drug products formulated within cocrystals of APIs are officially not yet marketed (apart from pharmaceutical salts being in their nature rather cocrystalline), but pharmaceutical industry has already adapted to large scale cocrystal production and formulation.

S3

STRUCTURAL CHARACTERIZATION OF GLYCERALDEHYDE DEHYDROGENASE FROM *THERMOPLASMA ACIDOPHILUM*

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Biotechnological production of chemical compounds is an environmentally more gentle process compared to their fabrication from natural fossil resources [1]. In terms of bio-production, cell-free processes are more effective than microbial production techniques since the enzymes can tolerate higher concentrations of final product than the cells. The glyceraldehyde dehydrogenase from *Thermoplasma acidophilum* (*Ta*AlDH) is a part of an artificial cell-free enzyme cascade for production of isobutanol and ethanol from glucose. *Ta*AlDH catalyzes the oxidation of D-glyceraldehyde to D-glycerate in this synthetic pathway [2]. Various mutants of *Ta*AlDH were constructed by random approach followed by site-directed and saturation mutagenesis in order to improve the enzymes' properties essential for its functioning within the cascade. Further optimization of *Ta*AlDH requires structural information about the enzyme for which crystallization followed by X-ray diffraction analysis was employed [3].

Diffracting quality crystals of *Ta*AlDH wild type were obtained after initial screening in condition H6 of the Morpheus screen (Molecular Dimensions Ltd., UK) followed by optimization, including variation of pH, protein and precipitant concentrations and ratios. Full data set was collected on the on the BL 14.1 operated by the Joint Berlin MX-Laboratory at the BESSY II electron-storage ring (Berlin-Adlershof, Germany) at 1.95Å resolution. Crystals belong to monoclinic $P2_{C1}$ space group with unit cell parameters of $a = 95.29$ Å, $b = 152.35$ Å, $c = 149.90$ Å, $\quad = \quad = 90.0°$, $\quad = 92.19°$. Matthews coefficient of $V_M = 2.41$ Å^3 Da^{-1} suggests that the crystals contain 8 molecules per asymmetric unit with a solvent content of 48.95 %.

The structure of *Ta*AlDHwt was solved by molecular replacement using the coordinates of betaine-aldehyde dehydrogenase from *Pseudoalteromonas atlantica* T6c (sequence identity 38%, PDB ID 3K2W). The final model contains two tetramers in the asymmetric unit that are related by non-crystallographic symmetry with differences observed in regions participating in crystal contacts. The *Ta*AlDHwt homotetramer consists of two homodimers that display a very tight connection through the formation of an extended beta-sheet between monomers of the dimer. The structure refinement of *Ta*AlDHwt is in progress.

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S4

CRYSTALLIZATION OF NOVEL HALOALKANE DEHALOGENASE FROM *GLACIECOLA AGARILYTICA* NO2 AND ATOMIC FORCE MICROSCOPY APPLICATIONS IN MACROMOLECULAR CRYSTALLIZATION

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The main bottleneck of solving protein structures by means of X-ray crystallography is obtaining good quality protein crystals. Atomic force microscopy (AFM) can provide information about growth and perfection of crystals, impurity effects, and defect formation, furthermore, AFM images can give low - resolution phase information which could enhance X-ray diffraction analysis (Malkin et al., 2004). This type of probe microscopy allows topography of soft biological samples to be imaged *in situ* in physiological condition with molecular resolution. Therefore combination of AFM and X-ray diffraction techniques will be used as a toolkit for macromolecular crystallography.

Microbial enzymes such as haloalkane dehalogenases have attracted significant interest because of their ability to catalyze the irreversible hydrolysis of wide range of halogenated compounds to the corresponding alcohol, halide ion and proton. These enzymes play an important role in biodegradation of halogenated compounds, which appear to be environmental pollutants (Newman *et al*., 1999, Janssen, 2004). Novel haloalkane dehalogenase DgaA was isolated from psychrophilic and moderate halophilic organism *Glaciecola agarilytica* NO2 found in marine sediment collected from the East Sea, Korea (Yong *et al*., 2007).

Microcrystals of DgaA grew in several conditions of JCSG-*plus* and Structure Screen 1&2 (Molecular dimensions Ltd, UK) after initial screening. Optimization including variation of salt, protein and polymer concentrations resulted in certain improvement of crystals. The best needle-shaped crystals with size about 0.8 0.039 mm and plate clusters with plate size of approximately 0.03 0.2 mm were obtained. Further crystals preparation for AFM experiments is in progress.

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S5

STRUCTURAL COMPARISON OF DpcA AND DmxA, -HYDROLASE FOLD FAMILY MEMBERS FROM DIFFERENT HLD SUBFAMILIES

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The enzyme DpcA from *Psychrobacter cryohalolentis* K5 and DmxA from *Marinobacter sp.* ELB17, catalyzing the hydrolytic conversion of halogenated aliphatic compounds with releasing a corresponding alcohol, a halide ion, and a proton as the reaction products belong to the haloalkane dehalogenases (EC 3.8.1.5; HLD), HLD-I and HLD-II subfamilies, respectfully. The enzymes are potentially practical for such applications as biodegradation, biosensing, protein tagging for cell imaging and protein analysis, decontamination of warfare agents, production of optically active hydrocarbons and alcohols.

DpcA and DmxA own unique temperature profiles with exceptionally high activities at low temperatures for DpcA (25 °C, pH 8.7, towards 1,3-dibromopropane) and height temperatures for DmxA (the maximal activity towards 1,3-diiodopropane was detected at 55 °C, pH 9.0), what highlights them among the other HLDs.

The crystallized crystals of DpcA diffracted to the resolution 1.05 Å, beamline 14.2 (BESSY II electron-storage ring, HZB, Germany), $P2_1$ space group and DmxA to the resolution 1.45 Å, beamline ID29, at the (ESRF, Grenoble, France), $P2_12_12_1$ space group.

The structures were solved by molecular replacement with *MOLREP* from the CCP4 software suite by using the coordinates of 1B6G (40% sequence identities and 53% sequence similarity) as search model for DpcA structure and 4E46 for DmxA (48% sequence identity and 63% sequence similarity).

The proteins have a globular shape and are composed of two domains: a highly conserved main domain, which is the scaffold - like for the catalytic residues, and a smaller helical cap domain, covering the active site, which has revealed the catalytic pentad essential for the activity of DpcA: Asp-123, His-280, Asp- 250, Trp-124,Trp-164 and for DmxA: Asp 105, His 273, Glu 129, Gln 40 , Trp106.

DpcA has one molecule in assymetric unit and DmxA – two, a uniquely formed by the covalent disulfide through Cys 294, the homo- dimer is chosen as biological asymmetric unit.

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S6

THE STUDY OF CE16 ACETYL ESTERASE FROM THE FUNGUS *HYPOCREA JECORINA*

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Fungus *Hypocrea jecorina* – teleomorph of *Trichoderma reesei* belongs to the saprotrophic filamentous fungi. The representatives of *Hypocrea* are known primarily by production of different cellulases and hemicellulases which are currently used in various fields of industry, mainly in paper processing and feed and food productions. As a part of the enzymatic cocktail secreted by *H. jecorina* during its growth on cellulose, also acetyl esterase was identified. Later it was classified as a first member of newly organized carbohydrate esterase family CE16 (CAZy classification). The enzyme is not capable of deacetylation of polymeric substrates as acetyl glucuronoxylan, but efficiently deacetylates terminally acetylated xylooligosaccharides [1] and shows different positional specificity on mono acetates, e.g. 4-nitrophenyl -D-xylopyranoside [2]. Further studies showed that the acetyl esterase is crucial for complete deacetylation of naturally acetylated xylans enabling their saccharification by xylanases. To study the relation-

ship between structure and function of acetyl esterase, highly purified recombinant enzyme produced by *Trichoderma reesei* Rut C-30 was prepared and crystallized. The enzyme is composed of 348 amino acid residues from which the first 19 form a secretion signal peptide. It was proved that the enzyme is glycosylated. The enzyme crystallized very easily, however, the crystals did not diffract. Later, after optimization, also diffracting crystals were obtained and a complete set of diffraction data was collected to 3.98 Å. After crystal annealing another data set was collected to 2.98 Å resolution using synchrotron radiation source at EMBL, DESY, Hamburg. Unfortunately, structure solution by molecular replacement method failed as there is no suitable model structure in PDB. Isomorphous crystals prepared by the native crystal soaking in the solution containing NaBr did not diffract at all. To make the future structure-function study of the enzyme easier, recombinant non-glycosylated enzyme should be prepared. For this purposes synthetic gene optimized for expression in *E.coli* was designed and synthesized.

The first non-glycosylated acetyl esterase obtained by the expression of its synthetic gene in *E.coli* cells was mostly insoluble or aggregated; moreover, even the soluble part was not able to bind to Ni-column. Conditions of cell cultivation, induction of gene expression, cells sonication and protein purification using metallo-chelate and size exclusion chromatography were necessary to optimized. At present, after optimization of all steps, the non-glycosylated recombinant CE16 acetyl esterase was prepared in the soluble and active form. All the results were confirmed by SDS-PAGE analysis, analytical FPLC, DLS study and by activity measurement. Preparation of the enzyme in the amounts sufficient for crystallization is under way.

Besides this, bioinformatic studies of the *Hypocrea jecorina* acetyl esterase as well as all other CE16 members (153 enzymes to this date) were performed. Bioinformatic studies proved that CE16 family members belong to the group of serine hydrolases, more exactly to the group of SGNH lipases/acetyl esterases [3]. They comprise highly conserved GDSL/GDSY motif, typical signature of many esterases and lipases. Alignment of amino-acid sequences showed the most conserved regions, mainly amino-acids of catalytic triad, Ser18, Asp295 and His298, and oxyanion hole, Gly92, Asn148 (part of binding site, numbering according to recombinant acetyl esterase). Also other conserved amino acids were identified; mainly tryptophans and phenylalanines which might be important for substrate binding. These assumptions have to be experimentally confirmed.

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Každoroční středoevropská konference biokrystalografů bude pořádána v ČR již počtvrté. Je primárně organizovaná Biotechnologickým ústavem AV ČR a Českou společností pro strukturní biologii. Předsedou je Jan Dohnálek.
www: http://crysa.fzu.cz/hec18/