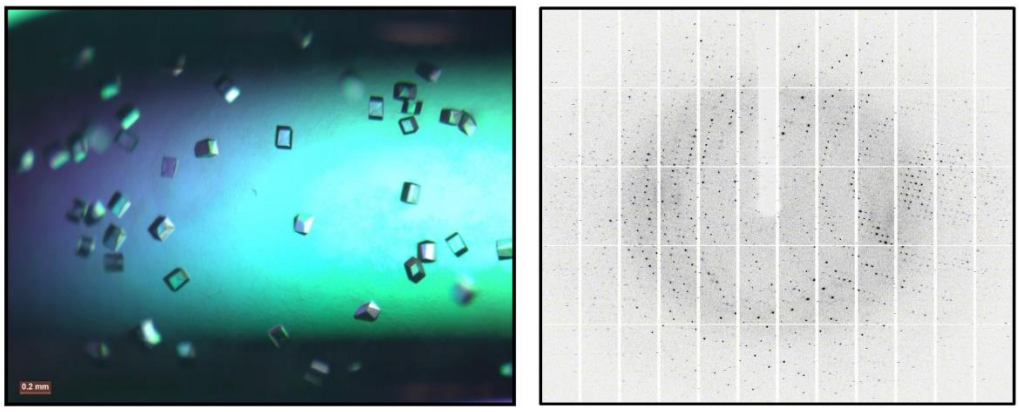
Implementation, achievements and opportunities

WP3

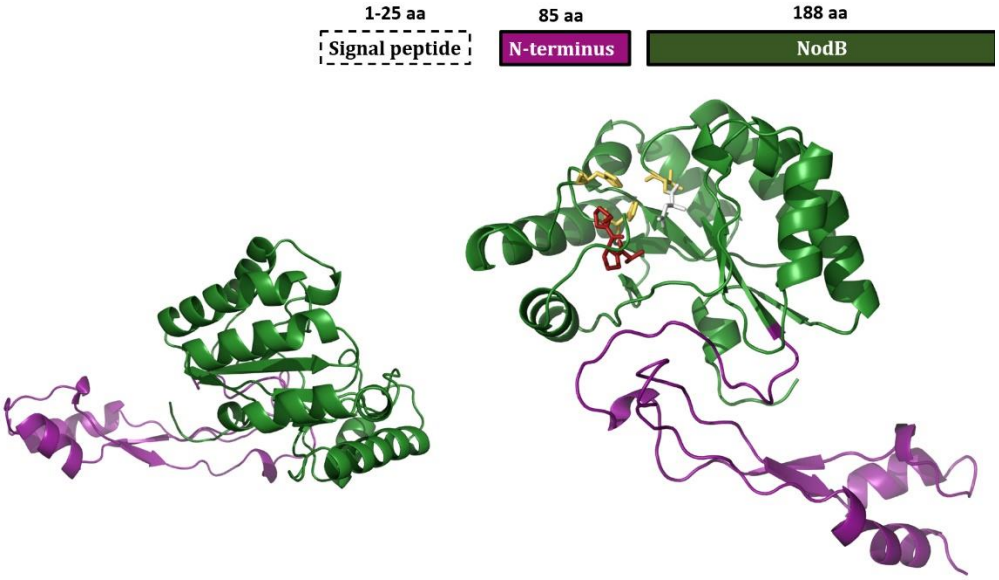
Structural studies of the pseudo enzyme B*a*3943 and its mutants

A systematic mutagenesis and crystallographic/ SAXS study has been performed for the pseudo enzyme B*a*3943. The crystal structures revealed details of the hydroxylation and active site characteristics, as well as the structural basis of the evolutionary path of the protein. These insights were exploited in the reverse-engineering the pseudo enzyme towards an active state, a process which beyond its scientific importance, has revealed essential components for catalysis and the Cα hydroxylation event. Three essential amino acid residue substitutions satisfy the necessary and sufficient requirements for the activation of the hydroxylation and catalytic mechanisms. Structure determinations at very high resolution has allowed insights into the roles of proline or glycine in hydroxylation and the intertwining of hydroxylation and deacetylation. The correlation between these two mechanism demonstrate the importance of hydroxylation as an active-site maturation process in the overall catalytic mechanism. The studies of B*a*3943 mutants provided structural information at very high resolution.

PROTEIN	Crystals Grown/ Data Quality Crystals	Diffraction Limit/ Structure determined
B*a*3943 (wt protein)	Y/Y	1.1 Å/ y
B*a*3943 N94D	Y/Y	1.5 Å/ y
B*a*3943 N94D V95D	Y/N	-
B*a*3943 N94D V95D A183R	Y/Y	1.7 Å/ y
B*a*3943 N94D V95D A183K	Y/N	-
B*a*3943 N94D V95D A183R P185G	Y/Y	1.2 Å/ y
B*a*3943 N94D V95D A183R Δ186 Δ187	Y/Y	0.9 Å/ y
B*a*3943 N94D A183R	Y/N	-
B*a3943* N94D V95N A183R	Y/Y	2.1 Å/ y
B*a*3943 N94D V95N A183R L235A	N/N	-
B*a*3943 N94D V95N A183R L235A	N/N	-



Ba3943 N94D V95D A183R: crystallization and collection of X-ray diffraction data



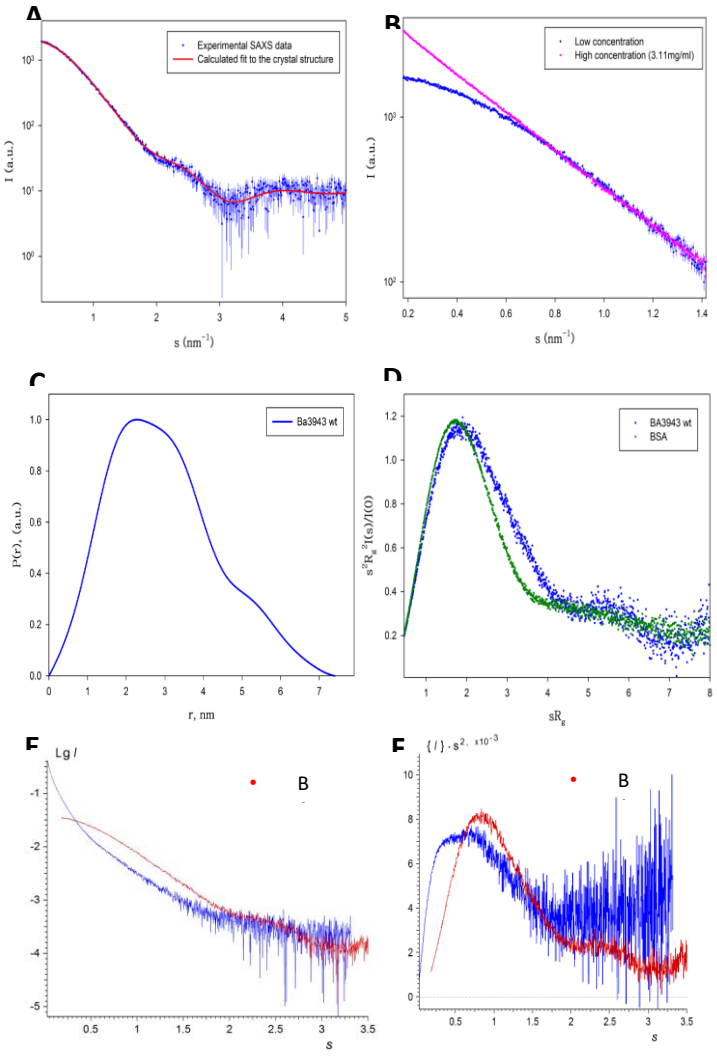
The Ba3943 N94D structure showing the NodB domain, selected residues of the active/ Cα hydroxylation site (N94D is enzymatically inactive) and the variable N-terminal region. The N-terminal regions are overwhelmingly missing from previous cystallographic structures of PDA family members due to excessive disorder.

*Ba*3943: SAXS analysis

Small Angle X-ray Scattering (SAXS) data were collected at the EMBL/DESY synchrotron site or at the beamline SWING of the SOLEIL synchtrotron for a range of temperatures and sample concentrations. The unique on-line size exclusion HP liquid chromatography facility at the SWING station improves dramatically the mono dispersity of the samples. Specialist software was used for data processing, calculation of particle dimensions, assessment of the folding states of the proteins and generation of structural models. CD spectra were recorded using a J-815 (Jasco Inc.) spectrometer or at the SOLEIL synchrotron (Gif-sur-Yvette, France).

Wild type B*a*3943 and an enzymatically restored mutant (B*a*3943_ N94D/V95D/A183R/L235D) were primarily measured with SAXS in order to assess the behavior of the proteins in solution and the agreement with the crystallographicaly derived structures. Wild type B*a*3943 (A) showed some aggregation tendencies at higher concentrations (B) and extrapolation to infinite dilution was performed to correct for these effects. The radius of gyration was estimated at 22.5 Å and the molecular weight was in agreement to that expected for a monomer. The distance distribution function P(r) (C) indicates a somewhat spherical structure with a disk-like anisometry. This is also supported by the Kratky plot (D) that shows a well-folded protein, albeit a bit more extended than a spherical protein such as BSA. We originally expected that the extended N-terminal beta-sheet domain of the protein will exhibit significant movement in respect to the NodB C-terminal domain, but, interestingly, the fit of the solution SAXS data to the crystallographically-derived structure of B*a*3943 is very good, indicating that this is not the case. On the other hand, the solution behavior of an enzymatically restored mutant was not ideal showing extreme aggregation effects even at low concentrations (E) and a Kratky plot indicating increase extendedness and

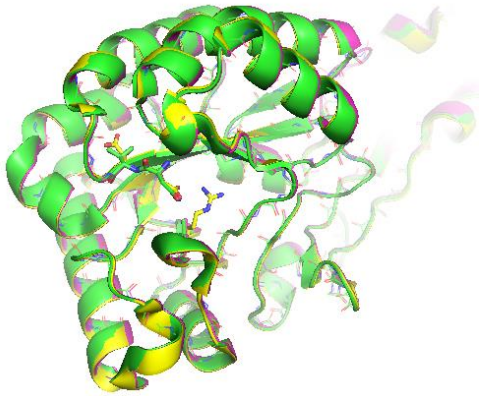
flexibility compared to the wild type protein (F) even though all mutations are not at the surface of the protein but at the “active site” of the protein.



SAXS analysis of Ba3943

The following of the proteins have been already deposited with the PDB

- Wild-type B*a*3943 (PDB id 7BKF)
- Mutant B*a*3943 N94D (PDB id 6HPA)
- Mutant B*a*3943 N94D V95D A183R, restored enzymatic/hydroxylation activity (PDB id 6HM9)



Superposition of mutants with the PDB codes 7BKF (magenta), 6HPA (green) and 6HM9 (yellow) shown the conversion from an inactive to an active (in terms of enzymatic and hydroxylation activities) protein.

- B*a*3943 N94D V95D A183R P185G
- B*a*3943 N94D V95D A183R Δ186 Δ187
- B*a3943* N94D V95N A183R

which were also determined at very high resolutions, have been also submitted to the PDB and will be also deposited after their validation by the database is completed.: