mutants of active site residues of AnAEst were determined. While the wild-type enzyme showed highest catalytic efficiency for naphthyl esters relative to phenyl esters, the R54G mutant displayed a 2.4 fold increase in catalytic efficiency for phenyl esters over naphthyl esters. The kinetic studies in conjunction with docking studies confirm the structural role of Arg54 and other active site residues in both substrate binding and catalysis.

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Keywords: enzyme, kinetics, esterase

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Structural insight into iron pathways in ferritin

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Ferritin directs the reversible biomineralization of iron. Crystals of apoferritin loaded, in aerobic conditions, with different amounts of $FeSO_4$ and $CuSO_4$ were studied by X-ray crystallography and the structure of the tripositive iron and bipositive copper adducts were determined at 2.7 Å and 2.8 Å resolution, respectively.

Anomalous diffraction experiments reveal the binding of the iron substrate to the ferroxidase site and to other sites in the protein, including a possible nucleation site for the iron mineral.

The metal coordination sphere at the catalytic site is redox dependent. The differences between iron and copper binding provide clues on the reaction mechanism and on the path of iron from the so-called C3 pore (entrance) to the C4 pore (exit into the cavity) through the catalytic site.

The crystallographic data combined with the previously reported results from NMR experiments, magnetic susceptibility measurements and other crystallographic determinations on different metal adducts [1], [2], [3], provide an updated model for the iron processing by ferritin.

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Structural comparison of the milk-clotting enzymes bovine and camel chymosin

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Chymosin is an aspartic protease that clots milk, thus initiating cheese formation. Cows are the major source for milk, but contrary to what would be expected, bovine chymosin is not the best milk-clotting enzyme known – camel chymosin is better[1]! This surprising observation initiated this project to determine the cause of this behaviour.

The camel chymosin obtained from expression in *Aspergillus niger* has been examined. Six variants have been separated using hydrophobic interaction chromatography. The variants differ with respect to glycosylation, activity, and other, at this point, unknown properties.

The structure of the double glycosylated camel chymosin has been solved. A good comparison with bovine chymosin requires the availability of a good experimental data set. However, models but no experimental data have been deposited for the previously available structures. Therefore the structure of bovine chymosin has been determined to a higher resolution (1.8 Å) than those previously available.

The overall fold of bovine and camel chymosin is similar, however camel chymosin is found in a self-inhibited state, in which the N-terminal blocks the binding cleft. The conformation commonly seen in other aspartic proteases appears to be destabilized by charge and sterical differences of the N-terminal. The resuspended crystals of camel chymosin show milk-clotting activity, hence other conformations are available, in which the N-terminal is located outside the binding cleft. Chymosin's interactions with its inhibitors and substrates are being investigated. The structure of bovine chymosin in complex with the aspartic protease inhibitor pepstatin has been solved. The structural results will be discussed in relation to the differences in the milk-clotting properties of bovine and camel chymosin.

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Unveiling the substrate-bound structure of a Baeyer-Villiger Monooxygenase

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The high specificity, efficiency, and "green" properties of biocatalysts make them increasingly attractive alternatives to conventional chemical catalysts. The Baeyer-Villiger monooxygenases (BVMOs), which catalyze the synthetically useful Baeyer-Villiger oxidation reaction, are a promising class of biocatalysts. The broad substrate spectrum of these flavoproteins makes them particularly suited for use in industry, allowing them to be engineered for specific applications. These enzymes have an FAD cofactor, and use molecular oxygen and NADPH to convert a ketone to an ester. In spite of a great deal of research characterizing these enzymes, most notably cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. NCIMB 9871, very little structural information is available. Thus far, one crystal structure of phenylacetone monooxygenase in complex with the FAD cofactor [1] and two crystal structures of CHMO from *Rhodococcus* sp. HI-31 in complex with both FAD and $NADP^+$ [2] have been published. These three structures revealed some of the overall domain movements that are required for BVMOs to function. In particular, a large rotation in the NADPH-binding domain was revealed to cause the sliding in of the

nicotinamide head of $NADP^+$, as well as allowing the formation of a putative substrate binding pocket [2].

These three structures have been instrumental in increasing our understanding of the mechanism, from a structural perspective, of the BVMOs. The critical structure of the substrate-bound form, however, has thus far remained elusive. As a result, a number of questions remain. These include how the enzyme can accommodate both $NADP^+$ and the substrate simultaneously, how the substrate specificity is determined, and which residues play key roles in stabilizing the various intermediates. As such, the substrate-bound structure is necessary to properly guide attempts to engineer these enzymes towards specific substrates.

We have solved a crystal structure of CHMO showing the first substrate-bound, catalytic conformation of any classical BVMO. This structure provides insight into the intricate movements that are integral to its reaction mechanism, involving the enzyme, its cofactors, and its substrate. Of note, a unique conformation of the $NADP^+$ cofactor creates an environment that allows for the formation of an active site pocket in close proximity to the flavin ring system. This allows the positioning of the substrate into a catalytically relevant location. A number of residues are implicated in stabilizing this conformation and maintaining the substrate in the active site until catalysis is completed.

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Keywords: oxygenase, mechanism, flavoenzyme

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Structural and functional studies of pseudomonas mesoacidophila MX-45 trehalulose synthase and trehalulose hydrolase

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Various diseases related to the over-consumption of sugar make a growing need for sugar substitutes. Sucrose is an inexpensive and readily available d-glucose donor, thus industrial potential for enzymatic synthesis of the sucrose isomers trehalulose and/or isomaltulose from sucrose is large. The naturally occurring structural isomer of sucrose, trehalulose is produced by sucrose isomerases. Two adjacent gene homologs, *mutA* and *mutB* from *Pseudomonas mesoacidophila* MX-45 have been characterized and demonstrated an activity on these sugars.

The enzyme MutA hydrolyzes the substrates trehalulose, isomaltulose, and sucrose into glucose and fructose, with a highest activity on trehalulose, whereas the enzyme MutB is a trehalulose synthase and catalyses the isomerisation of sucrose to mainly trehalulose. Since these genes are responsible for uptake and utilization of trehalulose and isomaltulose, sucrose or the reaction products may be involved in transcriptional regulation [1].

Mature MutB and MutA proteins displayed 52% sequence identity. These two enzymes belong to family GH13 as classified in the CAZy database.

We have recently cloned, purified, crystallized and solved the crystal structures of MutB and MutA.

Three-dimensional crystal structures, native- and mutant complexes of the trehalulose synthase MutB reveal an aromatic clamp playing an essential role in substrate recognition and in controlling the reaction specificity [2] and highlight essential residues for binding the glucosyl- and fructosyl-moieties [3]. These structures allowed defining the mode of action of this enzyme, providing important information on the recognition mode of sugars as well as different stages of the catalytic reaction mechanism [4].

Comparative studies highlighting the structural differences between trehalulose synthase and isomaltulose synthase, gave important information on the structural determinants responsible for the specificity of products formed by the sucrose isomerases. To complete and validate the information currently known, several mutants of the enzyme MutB are currently being studied. The new mutations affect the affinity of the enzyme towards its substrate and to change the specificity of formed products by native enzyme MutB. MutA is the first enzyme described that is able to hydrolyze trehalulose, and therefore might be useful for some industrial processes, e.g., treatment of sticky cotton fiber. The precise description of the reaction mechanism therefore should give valuable insights into the functioning of this enzyme.

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X-ray and neutron crystallographic structure-based mechanism of archaeal inorganic pyrophophatase from *Thermococcus thioreducins*

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Soluble inorganic pyrophosphatase from *Thermococcus thioreducins* (Tt-IPPase) has been crystallized in five unique metal or substrate complexes within monoclinic and rhombohedral space groups. As a result, the X-ray crystallographic structures of IPPase were determined revealing a 1) substrate-free Tt-IPPase with Ca^{+2} analyzed at room temperature (22°C) at 1.65Å (PDB ID 3R5V); 2) Ca^{+2}-bound IPPase complexed with the $P_2O_7^{-4}$ substrate at 1.44Å (PDB ID 3Q4W); 3) Ca^{+2}-bound IPPase complexed with the $P_2O_7^{-4}$ substrate at 1.35Å (PDB ID 3Q9M); 4) Mg^{+2} activated IPPase bound to hydrolyzed substrate at 0.99Å (PDB ID 3Q46); and 5) Mg^{+2}-bound IPPase complexed with SO_4^{-2} at 1.08Å (PDB ID 3Q5V). In addition we have determined the neutron crystallographic structure of substrate-free bound to Ca^{+2} at 2.50Å analyzed at room temperature (PDB ID 3Q3L). Based on these structural data, we were able to visualize and hypothesize the sequence of catalytic events associated with the hydrolysis of pyrophosphate substrate for the first time in an archaea system. We report a catalytic mechanism that involves an Asp in the active site serving as a principal acceptor for the deprotonation of a water molecule that bridges two metals. As a result, the formation of an attacking nucleophile OH^- toward the hydrolysis of pyrophosphate substrate is proposed. In addition the deprotonation of water may also