β-Alanine Synthase: One Reaction, Two Folds and Mechanisms

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Abstract

β-alanine synthase (βAS) is the third enzyme in the reductive pyrimidine catabolic pathway which is responsible for the breakdown of pyrimidine bases, including several anti-cancer drugs in higher organisms. We have solved the high resolution structures of two β-alanine synthases that perform the same reaction but have two very different folds and utilize different reaction mechanisms. The fold of the homodimeric βAS from the yeast *Saccharomyces kluyveri* (SkβAS) identifies it as a member of the Acy1/M20 family of metallopeptidases. Its subunit consists of two domains, of which the larger harbors a di-zinc centre crucial for catalysis, while the smaller domain mediates dimerization. We determined the structure of a productive substrate complex, the first for a dimeric member of the Acy1/M20 family. With this structure we show that a conformational change from an open state to a closed state is required for catalysis. Additionally the results of our site-directed mutagenesis studies, performed to identify residues involved in substrate binding and catalysis are discussed. These results support the role of E159 as a catalytic base and identify R322 as a key substrate-binding residue.

We have also characterized the structure of βAS from *Drosophila melanogaster* (DmβAS) which has been identified as a member of the nitrilase superfamily, with a characteristic αββα-sandwich fold and a conserved Cys-Glu-Lys catalytic triad. We have determined the three-dimensional structure in two different space groups, in P2_12_12 to 2.8 Å and in C2 to 3.3 Å resolution, respectively. The first crystal form has four molecules in the asymmetric unit, forming a homotetramer in a dimer-of-dimers arrangement. This homotetramer is assembled into an octamer with the molecule from an adjacent asymmetric unit in a helical turn-like assembly. The same octameric formation is found in the second crystal form, which has all eight monomers present in the asymmetric unit.
LIST OF PUBLICATIONS

I  Lundgren S, Gojković Z, Piškur J, Dobritzsch D.  
Yeast $\beta$-alanine synthase shares a structural scaffold and origin with dizinc-dependent exopeptidases. J Biol Chem. 2003 Dec 19;278(51):51851-62

II  Lundgren S, Andersen B, Piškur J, Dobritzsch D.  
Crystal structures of yeast $\beta$-alanine synthase complexes reveal the mode of substrate binding and large-scale domain closure movements. J Biol Chem. 2007 Dec 7;282(49):36037-47

III  Lundgren S, Andersen B, Piškur J, Dobritzsch D.  

IV  Lundgren S, Lohkamp B, Andersen B, Piškur J, Dobritzsch D  
The crystal structure of $\beta$-alanine synthase from *Drosophila melanogaster* reveals a nitrilase fold and an octameric helical turn-like assembly. Submitted to J Mol Biol. 2007 Nov
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LIST OF ABBREVIATIONS

aa amino acids
AMP adenosine 5’-monophosphate
ATP adenosine 5’-triphosphate
βAS β-alanine synthase
CoA coenzyme A
DNA deoxyribonucleic acid
DTT dithiothreitol
E.coli Escherichia coli
FAD flavin adenine dinucleotide
FMN flavin mono dinucleotide
GABA γ-aminobutyrate
GMP guanosine 5’-monophosphate
IMP inosine 5’-monophosphate
NAD+, nicotinamide adenine dinucleotide, and its reduced form
NADH
NADP+, nicotinamide adenine dinucleotide phosphate, and its reduced form
PAGE polyacrylamide gel electrophoresis
RNA ribonucleic acid
TMP thymidine 5’-monophosphate
Zn zinc

Amino acids are referred to by their three- or one-letter abbreviation, shown in the table below.

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<th>Alanine</th>
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Nucleotides

Nucleotides play key roles in a vast number of biochemical processes, and nearly all cells have both the *de novo* and the salvage pathway to satisfy the requirement for nucleotides. One of their important roles is their utilization as activated precursors for DNA and RNA synthesis. Nucleotide derivatives are activated intermediates in many biosynthetic pathways, for example UDP-glucose is a precursor of glycogen. One of the most widely used energy sources in biological systems is ATP, an adenine nucleotide. Adenine nucleotides are also components of three major coenzymes: NAD\(^+\), FAD and CoA. Another interesting role played by nucleotides is to serve as metabolic regulators, for example cyclic AMP which is a mediator of the action of many hormones.

![Figure 1. A deoxyribonucleotide with its three individual components labelled.](image)

Nucleotides have three characteristic components (fig. 1). The first is a nitrogenous base that is either a purine or a pyrimidine. The second is a pentose that is either a ribose or a deoxyribose, and the last is a (mono-, di- or tri-) phosphate. The purine base arises from aspartate, formate, glutamine, glycine and carbonate (fig. 2A). The assembly of the pyrimidine base ring is not as complicated as that of the purine base, it is synthesized from carbamoyl phosphate and aspartate (fig. 2B).

The biosynthetic pathways of both purines and pyrimidines are regulated by feedback control. In the biosynthesis of purine there are three major
feedback mechanisms regulating the de novo metabolism, and in all three it is the end products (AMP, GMP and IMP) that inhibit the enzymes and prevent them from performing their reactions. For the de novo synthesis of pyrimidine there is one feedback control, at the level of the first enzyme aspartate transcarbamoylase which is inhibited by the end product CTP. Biosynthesis of both the purine- and the pyrimidine nucleotides are complicated pathways with several sequential steps before they become nucleotides, which for example are used in the formation of DNA or RNA.

**Figure 2.** a) The biosynthetic origins of the purine ring atoms and of the b) pyrimidine ring.

Purine and pyrimidine bases are recycled by the salvage pathways as an important way of controlling the amount of free purine and pyrimidine bases that are constantly formed during the metabolic degradation of nucleotides. If this is not functioning correctly severe conditions can occur, as for example the Lesch-Nyhan syndrome. It is caused by overproduction of purines and leads to high levels of uric acid in all body fluids, which cause gout and moderate mental retardation.

**Pyrimidine degradation**

There are three pathways identified for pyrimidine degradation (Loh et al., 2006). The two main routes of pyrimidine degradation in microorganisms were established in the early fifties by several different groups including
Lara, Lieberman, Kornberg and others (Vogels et al., 1976). The reductive pathway (fig. 3) is a three-step reaction that degrades uracil and thymine to β-alanine and β-aminoisobutyrate. In 1953 Fink et al. found that the reductive pathway they had identified in rat also resembled that used by some bacteria. Later the pathway was also identified in calf, yeast, plants and other eukaryotes. The yeast strains have always been an exception, as it was observed by LaRue and Spencer in 1968, because most of them only could degrade cytosine and uracil but not thymine. Today, we know that the reductive pathway is found in most eukaryotes and only in some bacteria.

In 1952 three groups identified the second degradation pathway as an oxidative pathway in some bacteria (Vogels et al., 1976). In 2001 Soong et al. elucidated the pathway in more detail as the old results were based on studies done on crude enzyme preparations (Soong et al., 2001). They found that uracil or thymine is first degraded by uracil/thymine dehydrogenase, and thereafter barbiturase catalyzes the amidohydrolysis of barbituric acid to ureidomaloneic acid. The last step is performed by ureidomalonase where the ureidomaloneic acid is degraded to malonic acid and urea.

In 2006, a new pathway was identified by Loh et al. called the rut pathway (for pyrimidine utilization) in Eschericia coli K-12 (Loh et al., 2006). The pathway involves six gene products that degrade uracil to 3-hydroxypropionic acid and thymine to 2-methyl-3-hydroxypropionic acid, both of which are waste products for E. coli K-12. So far the intermediate steps of the six-reaction pathway are unknown. One reason why this pathway escaped earlier identification may be that it allows utilization of uracil and thymine as the sole nitrogen source at 19°C but not at 37°C, the temperature at which most studies were performed.
Figure 3. Overview of the three identified degradation pathways for uracil and thymine, adopted from (Piškur et al., 2007).
Reductive Pyrimidine Degradation

The reductive pyrimidine pathway in detail

β-Alanine synthase (βAS; EC 3.5.1.6), also called β-ureidopropionase or N-carbamyl-β-alanine amidohydrolase, catalyzes the third reaction of the reductive pyrimidine catabolic pathway, in which uracil and thymine are metabolized to β-alanine and β-aminoisobutyrate (fig. 4.), respectively. In this pathway dihydropyrimidine dehydrogenase (DPD; EC 1.3.1.2) reduces uracil and thymine into 5,6-dihydrouracil and 5,6-dihydrothymine. In the next step dihydropyrimidinase (DHP; EC 3.5.2.2) opens the pyrimidine ring, forming N-carbamyl-β-alanine and N-carbamyl-β-aminoisobutyrate. βAS hydrolyzes these compounds irreversibly to β-alanine and β-aminoisobutyrate, under release of ammonia and carbon dioxide (Wasternack, 1980, Piškur et al., 2007).

Figure 4. Reductive pyrimidine degradation pathway for uracil.

β-alanine can be utilized in four different ways (of which the three first are possible in human):
A) β-Alanine can be degraded by GABA aminotransferase (in human and other organisms) or β-alanine amino transferase (in S. kluyveri) to malonic semialdehyde.
B) β-Alanine can, through a metabolic pathway, be incorporated into carnosine or anserine by carnosinase.
C) β-Alanine can act as a neurotransmitter.
D) In several organisms β-alanine can be converted into (R)-pantothenate together with ATP and (R)-pantoate by pantothenate synthetase.
Significance in Humans

The pyrimidine catabolic pathway plays a key role in the synthesis of nucleotides and also in the regulation of the pyrimidine pool in the cell and thus helps to maintain a balanced supply of precursors for nucleic acid synthesis (Huang et al., 2003). Furthermore, the degradation of uracil is an important source of β-alanine in mammals (Traut et al., 1996). The pathway is the main clearance route for cytotoxic pyrimidine analogues such as 5-fluorouracil (5FU), which are widely used for the treatment of a variety of common tumors like colorectal, head/neck and breast cancer (Heggie et al., 1987). The second enzyme in the pathway, DHP, activates the prodrug dexrazoxane to its active form ADR-925, which is used to protect the heart against cardiotoxic side effects from anthracyclines used in chemotherapy (Schroeder et al., 2002). Pyrimidines play an important role in the regulation of the central nervous system and metabolic changes that affect the levels of pyrimidines may lead to abnormal neurological activity (Connolly et al., 1996)

van Kuilenburg et al. measured the activity of the enzymes involved in the pyrimidine catabolic pathway in 16 different tissues from a single healthy patient. Their results showed that the activity of DPD could be detected in all tissues examined, with the highest activity in the spleen and liver. The highest activity of DHP was present in kidney and liver. For βAS the highest activity was observed in the same organs (van Kuilenburg et al., 2006). These results demonstrate that most of the entire catabolic pathway is confined to the liver and kidney.

This pathway has been reported to catabolize 80% of the 5FU administered to cancer patients into fluorinated β-alanine, necessitating high standard drug doses (Heggie et al., 1987). Incorporation of the remaining 5FU into anabolic pathways induces anti-tumor and cytotoxic effects, primarily by inhibition of thymidylate synthase required for the de novo synthesis of dTMP.

In patients who are deficient in DPD or DHP, 5FU clearance is dramatically reduced and standard doses cause excessive toxicity (van Kuilenburg et al., 2003, van Kuilenburg et al., 1999). This issue is of significant importance due to the widespread use of 5FU and the high frequency of DPD deficiency, which has been estimated to be 2-3% in Caucasians and 8% in Afro-Americans. Aberrations in the DPYD gene explain about half of the cases of extreme 5FU-related toxicity (McLeod et al., 1998), but unfortunately they are mostly suspected only after toxicities have developed. The frequency of occurrence is probably high
enough to justify screening for DPD deficiency prior to 5FU-based chemotherapy as standard clinical practice. Varying DPD activity is also responsible for much of the observed inter- and intra-patient differences in 5FU pharmacokinetics, while elevated DPD activity in tumors caused by enhanced expression is a determinant of decreased sensitivity and resistance to 5FU (McLeod et al., 1998). To increase the efficacy of 5FU and to allow a more convenient oral administration, attempts have been made to achieve better control of 5FU pharmacokinetics by means of DPD inhibition (de Bono et al., 2001). Enzyme inhibition would optimize chemotherapy by attenuating unpredictable 5FU pharmacokinetic variability and suppressing a major factor of tumour resistance to 5FU. Drug doses could be significantly lowered and side effects caused by the putatively neurotoxic fluoro-β-alanine diminished. Currently, there are several oral fluoropyrimidine formulations containing DPD inhibitors under clinical evaluation (Milano et al., 2004).

**Structural studies on DPD and DHP**

DPD, the first and rate limiting enzyme in the reductive degradation of pyrimidines, is a homodimer of 2x111kDa (fig. 5). So far the recombinant pig enzyme is the only DPD that has been structurally determined (Dobritzsch et al., 2001). It has five domains with different folds and contains a FAD and a FMN binding site. Electrons are donated by NADPH and transferred from the FAD (NADPH)-binding site via four iron sulphur clusters to the FMN and substrate-binding site, where uracil and thymine are reduced to 5,6-dihydrouracil and 5,6-dihydrothymine. The dimerization of the enzyme is necessary for the electron transport to work, because each electron transport chain is composed of two iron sulphur cluster from each subunit (fig. 5).

DHP, the second enzyme of the pathway, is a homotetramer of 4x60 kDa (fig. 6) and has a two-domain subunit structure. The larger catalytic domain has an α/β-barrel fold with two zinc ions bound in the active site, and the smaller domain has a β-sandwich fold. The reaction mechanism for the reversible hydrolysis of the pyrimidine ring to N-carbamyl-β-alanine and N-carbamyl-β-aminoisobutyrate, respectively, is dependent on the two zinc ions. The three dimensional structure is so far known for the two eukaryotic enzymes, DHP from the yeast *Saccharomyces kluwyveri* and from the slime mold *Dictyostelium discoideum* (Lohkamp et al., 2006).
Figure 5. The dimer of DPD with the electron transport chain in black sticks, two of the iron sulfur clusters are from the neighboring subunit. Subunit A colored in dark gray and B in light gray.

Figure 6. The homotetramer of DHP with each subunit in a different shade of gray.
Aim of the Project

The main goal of the thesis project was to complete the structural model of the entire reductive pyrimidine catabolic pathway by determining the three-dimensional structure of β-alanine synthase. The pathway degrades one of the most widely used cancer drugs, 5-fluorouracil, and the availability of structural information for all enzymes is thus expected to give insights into the mechanism of drug clearance and to support structure-assisted design of inhibitors. To understand how the degradation of uracil and thymine is performed in chemical detail and to reveal organism-dependent differences, structural and functional studies were performed on β-alanine synthases belonging to two phylogenetically unrelated subfamilies.
β-Alanine Synthase

βAS from a historical perspective

In 1964 Dagg et al. performed in vivo activity measurements of the reductive pyrimidine pathway using different strains of mice to explore whether the same pathway was used for both uracil and thymine degradation. For a similar in vitro experiment the supernatant of homogenized rat livers was used (Dagg et al., 1964). Their hypothesis of uracil and thymine degradation by the same pathway was confirmed, and they also observed that some mice strains were slower in degrading pyrimidines than others.

βAS was purified to homogeneity for the first time in 1970 from livers from two different strains of inbred mice (Sanno et al., 1970). The enzyme was active and performed the last reaction in the degradation of uracil and thymine to β-alanine and β-aminoisobutyrate, with maximum activity measured around pH 7. An oxidative inactivation of βAS was also seen, which they could overcome with the addition of DTT.

In 1984 Traut et al. described a new thin-layer chromatography procedure that resolves the intermediates from the degradation of uracil and pyrimidine in rat liver in one dimension. Application of this method allowed easy preparation and detection of radioactive products and simplified kinetic measurements. In this study Traut et al. were able to show that the reaction pathway consists of three separate proteins and not of one larger protein complex.

The first article solely concerning βAS and not the complete pathway was published 1987 by Tamaki et al. (1987), who studied the rat enzyme in more detail. In the reported gel filtration experiments the enzyme eluted as a trimer of dimers. Kinetic measurements revealed the pH optimum of the reaction to be 7-7.5. Sigmoidal velocity curves not obeying Michaelis-Menten kinetics were observed, with Hill plots indicating substrate cooperativity. Tamaki et al. also measured the effect of several divalent cations on enzyme activity. The only effect they observed was a decrease of enzyme activity to 80% when adding 1mM Zn^{2+} to the reaction solution.

Matthews et al. further analyzed rat liver βAS in 1992 (Matthews et al., 1992). They agreed with Tamaki et al. that the native oligomer of the enzyme is a hexamer. Comparing the kinetic parameters V_{max} and K_{m} Matthews et al. clearly showed that the heat purification step used by Tamaki et al. damaged the enzyme and abolished allostERIC regulation. The molecular weight of the enzyme subunit was determined to 42 kDa +/- 2
and proposed to contain 398 amino acids. Furthermore, depending on the concentration of added substrate and product Matthews et al. could titrate the enzyme between a hexameric, a dodecameric and a trimeric state. From kinetic measurements ($K_m$ of 8 μM) it was deduced that the βAS reaction follows Michaelis-Menten kinetics in vivo.

In 1993 Kvalnes-Krick and Traut published a paper on rat βAS presenting the first DNA sequence of the enzyme, which contains 393 amino acids (Kvalnes-Krick et al., 1993). Other results suggested that rat βAS needs metal ions for its activity. Metal analysis was performed with native enzyme using optical emission spectroscopy, and yielded the presence of 1.8 equivalents of Zn$^{2+}$ per protein subunit. Further supportive evidence was a decreased enzyme activity when incubated with zinc-binding chelators. The authors also suggest the presence of two zinc binding motifs in the sequence of the enzyme after sequence comparison with other zinc binding enzymes. These results contradicted the earlier experiments performed by Tamaki et al.

The first βAS from an aerobic bacterium was purified from Pseudomonas putida in 1994 (Ogawa et al., 1994). The enzyme has quite different properties when compared with βASs from mammals and anaerobic bacteria. It is a 2x45 kDa homodimer with broad substrate specificity, which is dependent on divalent metal ions such as Co$^{2+}$, Ni$^{2+}$ or Mn$^{2+}$ for enzyme activity.

In 2001 three papers were published on different βASs (Walsh et al., 2001, Sakamoto et al., 2001 and Gojković et al., 2001). Walsh et al. describe βASs from plants (maize and Arabidopsis thaliana) and discuss, for the first time, a conserved cysteine residue as a possible nucleophile. They are puzzled about their results which indicate a Zn$^{2+}$-dependence of maize βAS activity, while no metal ion dependence of the enzyme activity was reported for a number of homologous enzymes like N-carbamyl-D-amino acid amidohydrolases (DCase). Inhibitor studies showed that the best inhibitors of maize βAS were simple aliphatic acids, which imply that βAS primarily recognizes the acidic portion of the substrate. Their characterization of βAS from maize with size-exclusion chromatography and native PAGE gave an estimated molecular mass of 440 kDa or large assemblies, suggesting a native oligomerization state of at least a decamer with subunits of 44 kDa.

A cDNA encoding βAS was isolated from human liver and expressed in E. coli by Sakamoto et al. The product, a 384 amino acid enzyme with a subunit molecular weight of 43 kDa, could be purified in sequential steps including a heating step, for which the lysate is heated to 50°C. The gel filtration pattern showed two peaks of active enzyme at 720 kDa and 380
kDa, consistent with a 16-subunit assembly and an octamer. They amended their earlier statement that the rat enzyme is a hexamer and now proposed that it is an octamer as well. The zinc content of the human enzyme was measured to be 0.54 atoms per subunit, but when they fed rats on a zinc-free diet for four weeks, they could not see any change in βAS activity between the control group and the zinc-free diet group, concluding that either the zinc ion is very tightly bound to the enzyme or zinc is not necessary for the activity of the enzyme.

Gojković et al. performed a multiple alignment and phylogenetic analysis of available carbamyl amidohydrolase sequences and could group the enzymes into three subfamilies. The majority of eukaryotic βASs belonged to one subfamily, with pairwise sequence identities of 55%. βAS from the yeast *Saccharomyces kluyveri* (SkβAS), which belongs to the second subfamily, shares only a limited sequence similarity with these enzymes, but is instead closely related to bacterial N-carbamyl-L-amino acid amidohydrolases (30–40% sequence identity). A third subfamily of amidohydrolases consists of bacterial and archaeabacterial DCases, with a pairwise sequence identity as low as 20% (Gojković et al., 2001). To confirm that *S. kluyveri* also has a pyrimidine catabolic pathway Gojković et al. took a pyd3 (pyrimidine degradation step three) mutant that was unable to grow on NCβA as its sole source of nitrogen and also exhibited diminished βAS activity. Gojković et al. placed putative PYD3 sequences from *D. melanogaster*, *S. kluyveri* and slime mold under the control of the *S. kluyveri* PYD3 promoter. These enzymes complemented the pyd3 defect, confirming the hypothesis. Through this experiment Gojković et al. also showed that these three genes are a part of the pyrimidine degradation pathway.

During the same year Pace and Brenner placed the majority of eukaryotic βASes as one branch in the nitrilase superfamily based on the conserved CEK motif (Pace et al., 2001).

The first crystal structure of a βAS was determined 2003 by Lundgren et al. and a high structural similarity of SkβAS to di-zinc dependent exopeptidases was revealed by a similarity search using the TOP server, especially to those from the M20 and M28 families.

In 2005 βAS from calf was characterized by Waldmann et al.. It is a hexamer in its native state with a subunit molecular mass of about 40 kDa, and no allosteric regulation of the enzyme was observed upon addition of substrate or product. The optimal enzymatic activity was obtained at pH 5.7 – 7.0 and the $K_M$ value for NCβA was 21.7 μM (Waldmann et al., 2005).
In conclusion, eukaryotic βASs have been purified and characterized from a number of sources including human, calf, rat, mice, maize, *Arabidopsis thaliana* and fruit fly. Their subunit molecular masses range from 42-50 kDa. Allosteric behavior has been observed for rat liver βAS, which changes the oligomeric state in response to effector molecules from a stable homohexamer to either an inactive trimer (in presence of the product β-alanine) or an active dodecamer (in presence of the substrate NCβA) (Matthews et al., 1987). It has been reported for two eukaryotic
βASs (rat and maize) that they display a decreased activity of the enzyme when incubated with zinc chelators, therefore suggesting the enzyme to be dependent on a divalent metal for activity, but it has also been reported for rat βAS that the activity decreases when Zn$^{2+}$ is added (Tamaki et al., 1987, Kvalnes-Krick et al., 1992, Walsh et al., 2001). Before solving the structures of the βASs belonging to the different groups, one could only speculate about the differences and similarities between the enzymes. As we succeeded to determine the structures of both fruit fly βAS (DmβAS) and SkβAS it is now clear that βASs from diverse organisms belong to two different fold classes that perform the same reaction, but with one subfamily using a metal-dependent and another using a metal-independent mechanism.

### β-alanine and β-aminoisobutyrate

GABA is the major inhibitory neurotransmitter in the mammalian brain and the actions of GABA are mediated by two different classes of receptors. The first class of receptors are GABA$_A$ which are ligand-gated channels that are selectively permeable to Cl$. The second class of receptors are GABA$_B$ receptors, which are G-coupled receptors that decrease cAMP levels and open K$^+$ channels. Drugs that modulate GABAergic activity, such as Vigabatrin and barbiturates, are effective in the treatment of a variety of neuropsychiatric disorders, in particular epilepsy and anxiety (Borden, 1996).

Glycine is another of the major inhibitory neurotransmitters in the adult mammalian central nervous system. A significant number of inhibitory synapses co-release glycine and GABA by the same pre-synaptic inhibitory neuron. This co-release probably happens as a consequence of the lack of discrimination between GABA and glycine of the vesicular inhibitory acid transporter VGAT/VIAAT. This transporter is localized in the pre-synaptic terminals of GABAergic and glycineergic neurons (Supplisson et al., 2002).

β-alanine is a structural analogue to glycine and GABA. There is evidence that indicates that β-alanine can also modulate neural activity in various parts of the central nervous system (Hadley et al., 2007, Wang et al., 2003, Sandberg et al., 1981) It has also been shown that β-aminoisobutyric acid is a partial agonist of the glycine receptor (Schmieden et al., 1999). Furthermore, β-alanine is a building block of carnosine and anserine, which are dipeptidic anti-glycation agents that have a putative role in
protecting the central nervous system against diverse types of pathology such as β-amyloid aggregation (Münch et al., 1997, Hipkiss et al., 1997). Bacteria, yeast and plants use β-alanine for biosynthesis of pantothenic acid (vitamin B5), while mammals attain vitamin B5 through their diet. Vitamin B5 is also an essential component of Coenzyme A and acyl carrier protein (Cronan et al., 1982). The betaine derivative of β-alanine works as the primary osmoprotectant in some plant families such as the Plumbaginaceae (Walsh et al., 2001). Through mutational studies of the pyd3 gene in Drosophila melanogaster it was revealed that the main source of β-alanine required for the pigmentation of cuticle comes from the pyrimidine catabolic pathway (Rawls, 2005).

**βAS deficiency**

So far, four cases of βAS deficiency have been reported from different countries. In the first case a girl of 17 months presented with muscular hypotonia, dystonic movements, scoliosis and severe developmental delay.

The second patient reported was a girl that had experienced an episode with high fever and meningitis at the age of six months. At eight months she suffered from afebrile seizures and showed a motor retardation of 2-3 months. After an MRI scan of the brain it was shown that the myelinization was delayed and the EEG showed presence of hypsarrhythmia. After one year of drug therapy with Vigabatrin the EEG did not show any epileptic activity and the treatment could be stopped. At the age of three years she still showed psychomotor retardation, severe mental retardation and a speech disorder, although visual contact appeared to be normal.

The third patient was a boy who was admitted to the hospital at the age of four months. He was febrile, alert and without respiratory abnormalities, but with a heart rate of 240 beats per minute. After two hours he went into an epileptic status that was hard to overcome and required generalized anesthesia. After some time the levels of lactate in the cerebrospinal fluid (CSF) normalized.

The last patient was hospitalized after an upper respiratory tract infection when she was 1 month and 10 days. In addition to a loss of consciousness for 30 min she was diagnosed with severe muscular hypertonia. After this she was examined but did not show any abnormalities. However, the
parents said that whenever she had a cold she would easily collapse (van Kuilenburg et al., 2004).

The reason for the βAS deficiency was revealed by analysis of the βAS-encoding PYD3 gene. Two splice-site mutations (IVS1-2A>G and IVS8-1G>A) and one missense mutation (A85E) were identified (van Kuilenburg et al., 2004). The latter enzyme variant was overexpressed in *E. coli* and showed no residual activity, hence explaining the βAS deficiency.

The level of β-alanine in the urine was determined to be the same for the βAS-deficient patients as for the control group, indicating that there is another source of β-alanine present and that the reason for their clinical state may not be the absence of β-alanine. There was also a therapeutic trial were oral supplementation of β-alanine was given to a patient without any convincible clinical improvement (Assmann et al., 2006), also indicating that it is not the loss of β-alanine that is the cause of the symptoms. So far there are no underlying mechanisms known that could cause the observed clinical symptoms, but there are speculations about oxidative stress or that the substrate N-carbamyl-β-alanine accumulates and either acts as a neurotoxin or inhibits the mitochondrial energy metabolism (van Kuilenburg et al., 2004).

There are about 50 patients reported with a dihydropyrimidine dehydrogenase defect and nine patients with a dihydropyrimidinase deficiency. The clinical symptoms were also here highly variable but centered around neurological problems (van Kuilenburg et al., 2006).
The Acy1/M20 family of enzymes

The aminoacylase-1/metallopeptidase 20 family (Acy1/M20) comprises enzymes with diverse metabolic functions. A few of these proteins have therapeutic significance. Bacterial DapE for example, is a potential target of antimicrobial compounds (Bienvenue et al., 2003) and functions in the succinylase pathway. Human serum carnosinase has been suggested as a promising drug target in the treatment of diabetes (Teufel et al., 2003, Janssen et al., 2005). Carboxypeptidase G2 from the Pseudomonas sp. strain RS-16 is currently under development as a rescue agent in cases of methotrexate overdoses (Widemann et al., 2006). Other members of this enzyme family are the bacterial allantoate amidohydrolase (AAH) involved in purine degradation, and bacterial PepV and PepT, which function in amino acid utilization (Jozic et al., 2002). The first described and name-giving enzyme of the family, mammalian acylase-1 (Acy1), functions in the salvage of Nα-acetylated amino acids from protein degradation (Lindner et al., 2003).

<table>
<thead>
<tr>
<th>Protein</th>
<th>PDB-ID</th>
<th>Catalyzed Reaction</th>
<th>Oligomerization</th>
</tr>
</thead>
<tbody>
<tr>
<td>βAS (S. kluyveri)</td>
<td>1r3n</td>
<td>degrades NCβA and N-carbamyl-β-aminoisobutyrate to β-alanine and β-amino isobutyrate</td>
<td>dimer</td>
</tr>
<tr>
<td>DapE (Neisseria meningitides)</td>
<td>1vgy</td>
<td>diaminopimelate biosynthesis, a critical precursor for cell wall and lysine biosynthesis</td>
<td>dimer</td>
</tr>
<tr>
<td>Serum carnosinase (human)</td>
<td>-</td>
<td>degrades the dipeptides carnosine and homocarnosine distributed in plasma and brain, a drug target in diabetes treatment</td>
<td>dimer</td>
</tr>
<tr>
<td>CPG2 (Pseudomonas sp.)</td>
<td>1cg2</td>
<td>hydrolyzes the C-terminal glutamate moiety from folic acid and its analogs such as methotrexate, a widely used anti-cancer agent.</td>
<td>dimer</td>
</tr>
<tr>
<td>Allantoate Amidohydrolase</td>
<td>1z2l</td>
<td>conversion of allantoate to (S)-ureidoglycolate, one of crucial</td>
<td>dimer</td>
</tr>
</tbody>
</table>
27

(E.coli K12) alternate steps in purine metabolism.

Pep V
(Lactobacillus delbrueckii) 1lfw a relative unspecific amino monomer peptidase, favors substrates with an N-terminal β-alanine or D-alanine residue, but also removes the N-terminal aa from a few tripeptides

Pep T
(Salmonella typhimurium) 1fno hydrolyzes tripeptides at their dimer N-termini, may contribute to the anaerobic utilization of amino acids as an energy source

Acylase-1
(human) 1q7l catalyzes the hydrolysis of dimer acylated L-amino acids to L-amino acids and acyl group

Members of the Acy1/M20-family exist as either monomers or homodimers. Several crystal structures for homodimeric members are available, e.g. those of CPG2 (Rowsell et al., 1997) and PepT (Håkansson et al., 2002). The subunit consists of two domains, a larger catalytic domain that contains all metal-binding and most putative substrate-binding residues and a smaller dimerization domain. All the zinc-binding amino acids are conserved within the Acy1/M20 family except for His226 in SkβAS and AAH, which is an acidic residue, either Asp or Glu, in the other enzymes (Table 2). The catalytic domain has a characteristic αβα sandwich architecture and the dimerization domain forms an antiparallel four-stranded β-sheet and two long α-helices. The sequence identities between the di-zinc dependent exopeptidases and other Acy1/M20 family members are only around 20% (Lundgren et al., 2003).

Table 2. Zinc coordinating residues

<table>
<thead>
<tr>
<th>Protein</th>
<th>PDB- ID</th>
<th>Zinc 1</th>
<th>Connecting</th>
<th>Zinc 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>βAS (S. kluyveri)</td>
<td>1r3n</td>
<td>His</td>
<td>Asp</td>
<td>Glu</td>
</tr>
<tr>
<td>Allantoate Amidohydrolase (E. coli K12)</td>
<td>1z2l</td>
<td>His</td>
<td>Asp</td>
<td>Glu</td>
</tr>
<tr>
<td>Pep V</td>
<td>1lfw</td>
<td>His</td>
<td>Asp</td>
<td>Glu</td>
</tr>
</tbody>
</table>

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<tr>
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</tr>
<tr>
<td>Pep V</td>
<td>1lfw</td>
<td>His</td>
<td>Asp</td>
<td>Glu</td>
</tr>
<tr>
<td>Protein Name</td>
<td>Code</td>
<td>Residue 1</td>
<td>Residue 2</td>
<td>Residue 3</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>(Lactobacillus delbruecki)</td>
<td></td>
<td>87</td>
<td>177</td>
<td>119</td>
</tr>
<tr>
<td>CPG2 (Pseudomonas sp.)</td>
<td>1cg2</td>
<td>His</td>
<td>Glu</td>
<td>Asp</td>
</tr>
<tr>
<td>Poker T (Salmonella typhimurium)</td>
<td>1fno</td>
<td>His</td>
<td>Asp</td>
<td>Asp</td>
</tr>
</tbody>
</table>
Nitrilase Superfamily

The nitrilase superfamily includes nitrilases, amidases, acyl transferases and N-carbamyl-D-amino acid amidohydrolases and they occur both in prokaryotes and eukaryotes. The enzymes use a wide variety of substrates, e.g. a range of nitriles, acid amides, secondary amides, N-carbamyl amides, and N-acylated N-terminals of polypeptides (Brenner, 2002). The nitrilase superfamily can be clustered into 13 branches that perform four types of reactions (Pace et al., 2001). A table of the different branches and their reaction types can be found below. (Table 3 and fig. 8) The reaction performed by branch 1 is the nitrilase reaction. The second type is the amidase reaction, which is the most common and is used in branches 2, 4, 7 and 8. The third type, utilized by βAS and DCase from branches 5 and 6, is the carbamylase reaction - a special kind of amidase activity. The fourth type of reaction is performed by branch 9 and consists of a fatty acid transfer from phospholipids to a polypeptide amino terminus.

<table>
<thead>
<tr>
<th>Table 3. The different branches present in the nitrilase superfamily</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Nitrilases</td>
</tr>
<tr>
<td>2) Aliphatic amidase</td>
</tr>
<tr>
<td>3) Amino-terminal amidase</td>
</tr>
<tr>
<td>4) Biotinidase</td>
</tr>
<tr>
<td>5) β-alanine synthase</td>
</tr>
<tr>
<td>6) Carbamylase</td>
</tr>
<tr>
<td>7) Prokaryotic NAD+ synthetase</td>
</tr>
</tbody>
</table>

The crystal structures of nine distant homologues have been determined (see table 4 for pdb ID and Z-scores). Three members of the nitrilase superfamily have been studied by electron microscopy, cyanide-degrading enzymes from *Pseudomonas stutzeri* and *Bacillus pumilus* (Sewell et al., 2005) and a nitrilase from *Rhodococcus rhodochrous* J1(Thuku et al., 2007). All members of the superfamily have a characteristic αββα fold and a conserved Cys-Glu-Lys catalytic triad. All structures form dimers producing an αββα-αββα sandwich. The sequence identity in the nitrilase superfamily is around 20%. The dimers are organized into different higher oligomeric states, such as tetramers, hexamers and even up to octadecamers. It is only within the microbial nitrilases that a helical organization of the assemblies has been observed.
Figure 8. Four different types of reactions carried out by nitrilase superfamily members. a) the nitrilase reaction performed by branch 1. b) The amidase reaction performed by branches 2-4, 7 and 8. c) The carbamylase reaction performed by branch 5 and 6. d) The amidase reaction in reverse is performed by branch 9. The figure is adopted from Pace et al., 2001.
Table 4. Determined structures of enzymes from the nitrilase superfamily

<table>
<thead>
<tr>
<th>Protein</th>
<th>PDB-ID</th>
<th>Z-score</th>
<th>r.m.s.d. (Å)</th>
<th>Nr. aligned Cα-atoms</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCase (Agrobacterium sp.)</td>
<td>1erz</td>
<td>11.2</td>
<td>1.70</td>
<td>257</td>
<td>Nakai et al., 2000</td>
</tr>
<tr>
<td>DCase (A. radiobacter)</td>
<td>1fo6</td>
<td>10.9</td>
<td>1.68</td>
<td>258</td>
<td>Wang et al., 2001</td>
</tr>
<tr>
<td>PH0642 (P. horikoshii)</td>
<td>1j31</td>
<td>10.7</td>
<td>1.45</td>
<td>240</td>
<td>Sakai et al., 2004</td>
</tr>
<tr>
<td>XC1258 (X. campestris)</td>
<td>2e11</td>
<td>9.2</td>
<td>1.55</td>
<td>229</td>
<td>Chin et al., 2007</td>
</tr>
<tr>
<td>Nit3 (S. cerevisiae)</td>
<td>1f89</td>
<td>9.1</td>
<td>1.57</td>
<td>232</td>
<td>Kumaran et al., 2003</td>
</tr>
<tr>
<td>amiF (H. pylori)</td>
<td>2dyu</td>
<td>8.9</td>
<td>1.95</td>
<td>253</td>
<td>Hung et al., 2007</td>
</tr>
<tr>
<td>NitFhit (C. elegans)</td>
<td>1ems</td>
<td>8.7</td>
<td>1.75</td>
<td>245</td>
<td>Pace et al., 2000</td>
</tr>
<tr>
<td>amidase (P. aeruginosa)</td>
<td>2uxy</td>
<td>8.3</td>
<td>1.79</td>
<td>248</td>
<td>Andrade et al., 2007</td>
</tr>
<tr>
<td>amidase (G. pallidus)</td>
<td>2plq</td>
<td>8.0</td>
<td>1.83</td>
<td>245</td>
<td>Agarkar et al., 2006</td>
</tr>
</tbody>
</table>
SACCHAROMYCES KLUYVERI β-ALANINE SYNTHASE

Papers I and II concern the structure, kinetic parameters and reaction mechanism of βAS from the yeast S. kluyveri (SkβAS).

Paper I

Structure determination of βAS from S. kluyveri

Phasing

Paper I describes the structure determination as well as the crystal structure of SkβAS, an enzyme that contains 455 amino acids per monomer. Dobritzsch et al. described protein purification and crystallization of SkβAS in an earlier paper. Several data sets had been collected, both from native protein crystals and from different heavy atom derivative crystals (Dobritzsch et al., 2003). Molecular replacement and MIR phasing were tried without any success, most likely because of the low sequence identity to its closest homolog of known structure (<20 %), and non-isomorphism between the derivatives and native crystals.

After successful incorporation of selenomethionine (Se-Met), the substituted protein could be crystallized under the same conditions as the native protein. Complete datasets could be collected at three different wavelengths. A first phase model and initial electron density maps were obtained using just the peak data set. The structure was solved in the monoclinic space group P2₁, containing a homodimer in the asymmetric unit. The native protein crystallized in a different crystal form, belonging to the same spacegroup P2₁ but exhibiting a larger unit cell with a volume of about four times that of the Se-Met protein crystals. An early model of the SeMet-substituted SkβAS was used to phase the native data by means of molecular replacement using the program EPMR (Kissinger et al., 1999). The molecular replacement was divided into two different searches to account for potential differences in domain orientations (i.e. varying angles between the catalytic and dimerization domains) which were observed in SeMet-SkβAS. In the first round of molecular replacement a dimer of dimerization domain was used as a search model, while in the following run the catalytic domain was used. Four dimers were ultimately located in the asymmetric unit.
Figure 9. Overall structure of SkβAS dimer. The zinc ions are colored black, subunit A is in dark gray and subunit B is colored by its secondary structure, β-strands are in black and α-helices are in light gray.

Overall structure

The SkβAS monomer can be divided into two domains, which both have an α/β-fold (fig. 9) (Lundgren et al., 2003). The smaller domain (residues 247-365) mediates most of the inter-subunit contacts within the homodimer and consists mainly of two long α-helices packed against a four-stranded antiparallel β-sheet. The core of the larger catalytic domain (residues 1-246, 366-455) consists of a mixed three-layer α/β/α-sandwich.

Pairwise superpositions of the monomers present in the asymmetric unit of SkβAS crystals shows that due to a rigid body movement of the catalytic domain relative to the dimerization domain the structures are not entirely equivalent. This shows that the enzyme has an inbuilt conformational flexibility.

A high structural similarity of SkβAS to di-zinc dependent exopeptidases was revealed by a similarity search using the TOP server, especially to those from the M20 and M28 families. The catalytic domain of SkβAS superimposes well with the single domain-aminopeptidases from Streptomyces griseus (SGAP) and A. proteolytica (APAP). Both the catalytic and the dimerization domains of SkβAS superimpose well with the two-domain enzyme structures available for the M20 family (CPG2, PepT, AAH). For the monomeric member PepV, the lid domain has about the same size and fold as a dimer of dimerization domains in SkβAS (fig . 10b)
Figure 10. a) The CPG2 dimer colored by the subunits A and B, the zinc ions are colored black. b) The PepV monomer colored by its secondary structure, with an inhibitor bound in the active site.

**Metal-binding site**

The initial electron density map showed two strong peaks in close proximity to each other, indicating the presence of a di-metal center. The metal present in the center was proposed to be zinc ions, inferred from the coordinating residues and the high structural similarities to the di-zinc
dependent exopeptidases. This was later confirmed by an X-ray absorption scan at beamline ID-29 of the European Synchrotron Radiation Facility. The reaction mechanism in the Acy1/M20 family involves the activation of a water molecule by a di-metal center, before a nucleophilic attack on a carbon-nitrogen bond is performed. The location of the active site and the presence of a di-metal center in SkβAS were unknown prior to the determination of the three-dimensional structure. Based on this study we suggested that the active site contains a di-zinc center and is located in the vicinity of the inter-domain cleft.

![Figure 11. Zinc coordinating residues in SkβAS.](image)

The zinc coordination is illustrated in figure 11. Asp125 is bridging the two zinc ions. The moderate data resolution (2.7 Å) makes it impossible to determine whether there is also a water or a hydroxyl moiety bridging the two zinc ions. Observed B-values and distances and the electron density map calculated after refinement of a model containing such a bridge support the presence of a water or hydroxyl molecule. Other residues coordinating the zins are His114 and His226 (Zn1) and Glu160 and His421 (Zn2). These residues are all conserved in the Acy1/M20 family except His226, which is replaced by an acidic residue (Asp or Glu) in other proteins of the family (Table 2).
Substrate-binding residues and proposed catalytic mechanism

After comparison to the inhibitor binding in PepV and modeling of the substrate into the active site of SkβAS, potential residues involved in substrate binding were identified. For instance, Glu159 can, with minimal movements, bring its carboxylate group within hydrogen bonding distance to the amino group of the substrate as well as to the bridging water molecule. Glu159 is strictly conserved in all Acyl/M20 family members and is hydrogen-bonded to the metal-bridging water in all those of known structure. Three residues from the dimerization domain are most likely involved in the binding of the carboxyl group of the substrate, Arg322, His262 and Asn309. Residue Arg322 belongs to the same subunit as the respective catalytic domain, while His262 and Asn309 belong to the other subunit in the dimer.

When modeling the carbamyl moiety of the substrate in hydrogen bonding distance to the zinc-center, the distances from the carboxyl group to the putative carboxyl group-binding residues are too large to allow hydrogen-bonding in most of the monomers. These distances vary from 2.5 Å (chain B) to 3.7 Å (chain A) as the size of the cleft between the catalytic and the dimerization domain is different in the diverse subunits. This observation led to the conclusion that the catalytic domain moves closer to the dimerizaton domain upon substrate binding, thus properly positioning NCβA in the active site of SkβAS as part of the catalytic mechanism.

We proposed a reaction mechanism that is composed of six steps. 
Step 1, binding of NCβA to the carboxylate-anchoring residues Arg322, His262 and Asn309 in the active site.
Step 2, rigid body domain movements occur allowing simultaneous interaction of the substrate with the di-zinc center, displacement of a zinc-bound water by the substrate and activation of the bridging water molecule by Glu159.
Step 3, nucleophilic attack of the carbonyl carbon of the substrate by the activated water molecule and formation of the tetrahedral intermediate.
Step 4, cleavage of the carbon–nitrogen bond, β-alanine formation and spontaneous decomposition of the generated carbamate.
Step 5, opening of the active site and release of carbon dioxide and ammonia.
Step 6, release of the reaction product β-alanine and addition of a new bridging water molecule.
As discussed above there is one zinc-coordinating residue that is different in SkβAS (His226) and AAH (His192) than in other family members e.g. PepV (Asp177), CPG2 (Glu200) and PepT (Asp196). This replacement of an acidic residue with a histidine results in a decreased negative charge from the protein ligands, which most likely stabilizes the metal bound hydroxide. As a consequence, the zinc-bound water molecule in SkβAS should exhibit a lower pKₐ and higher reactivity than the metal bound solvent in the exopeptidases. This may be required for cleavage of the more resonance-stabilized and thus more stable N-carbamylated substrates, in comparison to cleavage of a peptide-bond.

Conclusion

We succeeded with the determination of the 3D-structure of SkβAS. Structural alignments revealed that the overall structure resembled that of di-zinc dependent exopeptidases. We therefore suggested a common origin for SkβAS and dizinc-dependent exopeptidases. The location of the active site as well as putative substrate binding residues could be identified. A reaction mechanism was proposed that includes an opening and closing of the active site cleft during each catalytic cycle.

Paper II

Large-scale domain movements and substrate binding

Site-directed mutagenesis and crystallization of SkβAS mutants

Since no SkβAS structure in complex with its substrate or product was available, we had proposed putative substrate binding residues identified by comparison with structurally related enzymes from the Acy1/M20 family. With the experiments resulting in the second publication we wanted to test our hypotheses by mutating some of the residues that we suggested to be important for substrate binding or to play a key role in the proposed reaction mechanism.

We produced six single mutants: E159, believed to be the catalytic base, was exchanged either to the shorter
Asp, which preserved the charge and the functional group, or to Ala for complete removal of the active group.

R322 and H262 are residues proposed to anchor the substrates carboxyl group - both were mutated to Ala to remove the substrate binding functional groups.

H226 was replaced by a Glu to mimic the di-zinc center coordination observed in most dizinc-dependent exopeptidases.

H397, thought to stabilize the transition state during the reaction, was mutated to Asn, a non-protonizable aa with similar side chain length and chemical properties as His.

All mutants were expressed and purified in the same way as the native protein (Lundgren et al., 2003). The original crystallization condition generated diffraction-quality crystals of only the E159A, E159D and R322A mutants. SkβAS E159A and –D did crystallize in space group P2₁ with the same unit cell dimensions as for the SkβAS SeMet-substituted protein, with one homodimer present in the asymmetric unit. The R322A crystals belong to space group P1, with four subunits in the asymmetric unit.

A new crystallization condition was found at a higher pH 8.75 (original pH 6.0-6.5) after a subsequent round of sparse-matrix and grid screening using nanotechnology. After optimization, diffracting crystals could be obtained in this condition for the native enzyme and four of the six mutants, all in space group P2₁ with consistent unit cell dimensions, but different to those earlier obtained under the original crystallization conditions.

Because of the failure to crystallize H226E, a thermal shift (Thermoflour) assay comparing the melting temperature of native and H226E-SkβAS was performed. The melting curves were almost identical for the two enzymes under all tested conditions, which implies that the failure in crystallization is not due to an overall structural instability of the mutant.

**Overall structure**

The backbone structure of the domains is largely conserved between the native and mutant SkβAS structures. However, a large conformational change of the overall structure is observed in the E159A mutant in complex with NCβA (E159A_NCβA) and the wildtype enzyme in complex with β-alanine (WT_βAla). Both of these represent the closed conformational state of SkβAS. Crystals obtained with the original
crystallization condition still produced structures of the open state, e.g. for R322A and E159A. Crystallographically independent closed subunits superimpose well with an r.m.s.d of 0.12 Å. As observed earlier in the native SkβAS open state structures, the angle relating the two domains varies between the different subunits due to conformation flexibility and does not align as well as the closed subunits.

The closed state

SkβAS is the first enzyme in the Acy1/M20 family for which a closed state has been crystallized and structurally characterized. However, a closed conformation has previously been proposed and modeled for several family members (Agarwal et al., 2007, Lindner et al., 2005, Vistoli et al., 2006).

Figure 12. a) The closed conformation of SkβAS. b) The open conformation of SkβAS.

When comparing the structure of R322A (open) to that of E159A_NCβA (closed), the catalytic domain makes a ∼30° rotation relative to the dimerization domain, leading to active site closure (fig. 12). When the active site is closed, the area buried between one catalytic domain and the remaining part of the dimer is about twice as large, ∼2500 Å², compared to ∼1200 Å² in the open state.

The magnitude of the movement is well illustrated by residue D192 at the tip of the large loop protruding from the catalytic domain. When the enzyme is in its open state, these residues (one from each subunit) are separated by a distance of ∼32 Å, while they are in van-der-Waals contact in the closed conformation.

The importance of the global conformational change is confirmed by the observation that the three residues (R322, H262 and N309) move into the
active site, and are properly positioned for substrate binding and catalysis.

**Metal-binding properties**

The metal center in SkβAS is situated in the active site cleft, with five residues coordinating two zinc ions, commonly termed zinc1 and zinc2. Previously determined structures of native and SeMet-SkβAS showed full occupancy for both zinc ions, although only 1 nM ZnCl$_2$ was added during protein growth and purification procedures. This indicates a stable binding of the two metal ions at pH 6.0 and 6.5, where the activity of the enzyme is only 20-30% of its maximum. It was therefore a surprise when the electron density maps for E159A_NCβA showed full occupancy for only zinc1, while zinc site 2 was occupied in only one third of the active sites in the crystal obtained at pH 8.75. Other data suggest that this is not an effect of removing the negative charge from the active site vicinity since similar zinc occupancies are observed in the WT enzyme under these conditions and full occupancy of the zinc center is seen at pH 6.0 for E159A. Substrate binding is not hampered by this phenomenon, since there is full occupancy observed for NCβA in all the active sites present in the asymmetric unit of the crystal. An interesting question is therefore whether this loss of the metal ion in two thirds of the active sites is just an artifact arising from the purification and crystallization, or if it is of functional significance. At this point there is no conclusive evidence for any functional significance of a mono-metalated enzyme form, or for an influence of the crystallization condition on the occupancy of the metal center.

**Substrate/product binding**

The inactive mutant E159A was co-crystallized with the substrate NCβA. After initial refinement with the ligand-free SkβAS model, there was positive electron density observed in the active sites resembling the shape of the substrate. NCβA was modeled into the active sites and refined well. It forms a salt-bridge and seven hydrogen bonds to residues R322, H262, N309, G396 and Q229 as well as a direct coordination to zinc2. Since the residues H262 and N309 are engaged in substrate binding within the partner subunit, the dimerization of SkβAS is necessary for the enzyme to form a complete active site and to be able to perform its reaction.
**Figure 13.** The substrate NCβA bound in the active site of the E159A mutant, hydrogen bonds are shown as dotted lines. The observed roles of the different residues are described beside the amino acids.

**Figure 14.** Superposition of the active sites of E159A_NCβA (carbon atoms in orange), WT-βAla (cyan), and the previously published wildtype open structure (white). Zinc ions in blue belong to WT_βAla, the single zinc ion in black to E159A_NCβA. The bridging water/hydroxyl moiety found in WT_βAla and the native enzyme is represented as a red sphere. The tetrahedral reaction intermediate (green carbon atoms) was modeled into the active site.
The identity of the amino acids involved in substrate binding had been proposed previously, although their roles had not been correctly assigned in all cases. For example, residue H262 was believed to be involved in the binding of the carboxyl group of the substrate, but it was observed that upon closure of the active site it is moved further in and binds instead to the carbamyl group.

When comparing the structures of the two complexes E159A_NCβA and WT_βAla, the interactions with the product β-alanine are homologous to those made to the corresponding atoms of the substrate in the E159A_NCβA complex. β-alanine is only present in two of four active sites in the WT_βAla complex, while the substrate is present in all four. The same concentration was used for both ligands during crystallization, indicating that the affinity for the substrate is higher than for the product. Since all four active sites are closed in the asymmetric unit, it seems that the crystallization condition stabilizes the closed conformation of the structure rather than the ligand binding in this case.

**Observed functional and structural effects of the mutations**

At low substrate concentrations SkβAS follows Michaelis-Menten kinetics, but exhibits substrate inhibition at concentrations higher than 100 mM NCβA. This behavior complicates the precise determination of $K_M$ and the constant is also somewhat dependent on the enzyme batch. The $K_M$ was determined to 50-70 mM for the native enzyme using NCβA as substrate, indicating low substrate specificity. The mutants had even higher $K_M$ values and most of them had very low residual activity. We therefore only present $k_{cat}$ and the relative activities of the mutated enzymes in comparison with WTSkβAS. (Table 5).

<table>
<thead>
<tr>
<th>Table 5. Kinetic analyses of wild type and mutant SkβAS</th>
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<tr>
<td><strong>Enzyme</strong></td>
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<td>------------</td>
</tr>
<tr>
<td>WT</td>
</tr>
<tr>
<td>E159A</td>
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The overall structure and the active site architecture are intact after the exchange of E159 to an Ala or Asp, but the activity of the mutated enzymes is dramatically decreased. The same drastic loss of activity is seen for the exchange of R322 to an Ala. In the structure of R322A NCβA binding was observed at an artificial binding site created by the deletion of the Arg side chain. No activity was detected for H226E, which is the only mutant for which no crystals could be obtained. Melting curves recorded for native SkβAS and the mutant H226E did not indicate any change in overall structural stability. Both H262A and H397N exhibit a 10-15 fold decrease in $k_{cat}$, but only H397N shows a 2-3 times increase of the $K_M$, implying that the substrate affinity is only affected by the exchange of H397 to Asn.

### Mechanistic implications

The enzyme activity is almost completely lost when E159 is replaced by an Ala or Asp, which is consistent with its crucial role as a general base in the proposed reaction mechanism. The lack of activity for the E159D mutant may be explained by the shorter amino acids inability to reach both the water molecule and the amide group of the substrate, to abstract and transfer a proton. Also the inactivity of R322A is consistent with our hypothesis and other biochemical data collected for Acyl/M20 family members, indicating that either the substrate is not bound at all by the mutant, or that it is bound in a way that makes the nucleophilic attack on the carbon-nitrogen bond impossible.

Because of the lack of structural data for H226E, it can only be speculated that perhaps the ability to coordinate zinc1 was lost and that this residue is indeed as important for activity as it has been proposed. Based on the open conformation of SkβAS, we previously suggested that H262 was involved in binding the carboxyl group of the substrate. Recent studies on Acyl mutants showed a dramatic decrease in $k_{cat}$ and increase in $K_M$ when H206

<table>
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<tr>
<th></th>
<th>$k_{cat}$</th>
<th>$K_M$</th>
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<tr>
<td>E159D</td>
<td>$5.0 \times 10^{-3} \pm 9.0 \times 10^{-4}$</td>
<td>0.09</td>
</tr>
<tr>
<td>R322A</td>
<td>$7.7 \times 10^{-3} \pm 1.0 \times 10^{-3}$</td>
<td>0.14</td>
</tr>
<tr>
<td>H262A</td>
<td>$0.472 \pm 2.3 \times 10^{-2}$</td>
<td>8.9</td>
</tr>
<tr>
<td>H226E</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>H397N</td>
<td>$0.383 \pm 2.4 \times 10^{-2}$</td>
<td>7.2</td>
</tr>
</tbody>
</table>
Figure 15. The corrected and extended reaction mechanism for SkβAS.

(corresponding to H262 in SkβAS) was replaced by an Asn or Ala. Structural analysis of E159A_NCβA does not contradict the kinetic data obtained for Acy1, even though the changes in k_{cat} and K_M are not as pronounced for H262A in SkβAS. They support that this residue is more important for catalysis than for substrate binding. It is surprising, however,
that the exchange of His397 to Asn has a larger impact on the catalytic efficiency than H262A. Since we lack structural data for the closed conformation of H397N we can only assume that active site interactions of this residue are important for correct placement and orientation of the substrate and/or for maintenance of the metal center geometry. Based on the accumulated data on SkβAS we suggest that the open and closed conformations coexist in a dynamic equilibrium, with the open state allowing ligand binding and the closed state promoting catalysis. A revised mechanistic proposal is depicted below.

**Conclusion**

We have determined the structure of the first closed state conformation for a member of the Acy1/M20 family. Residues proposed to be important for substrate binding or catalysis were mutated to either Ala or amino acids with different properties than the original. The mutant enzymes were characterized, allowing correction and extension of the proposed reaction mechanism.
Drosophila Melanogaster βAS

Papers III and IV concern the crystallization and structure determination of Drosophila melanogaster β-alanine synthase (DmβAS). A reaction mechanism is also proposed.

Paper III

Crystallization of βAS from D. melanogaster

Protein expression and purification

We intended solve the first crystal structure of a higher eukaryotic βAS. βAS from Drosophila melanogaster was recombinantly expressed with a C-terminal (His)$_8$-tag in E. coli, using the same growth and purification protocol as for SkβAS (Gojcović et al., 2001). The calculated subunit molecular mass for DmβAS is 46.1 kDa (386 amino acids +19 tag residues).

Crystallization

Several commercially available grid and sparse matrix screens were used in our attempts to crystallize DmβAS, using the vapor diffusion method in sitting drops at room temperature. During the first rounds of screens, no crystalline material was obtained. These setups were repeated with different additives and combinations of additives present in the protein solution. After one month of equilibration, small spherical crystals were found in two conditions in the Wizard I and II screens (Emerald Biosystems). A combination of two additives was necessary for crystal growth, the substrate 10mM NCβA together with 5% glycerol. The crystallization condition was further optimized in 24-well plates using the hanging drop method and crystals were now obtained after 14-30 days. The crystallizations drops went through some sort of metamorphosis: they stayed clear for a few days and then a glistening microcrystalline precipitate was formed. This precipitate then acquired a mesh-like structure after another 2-3 days. Crystals appeared in about half of the drops from this structured precipitate, but only 10% of those crystals were good enough for X-ray diffraction. Attempts to further optimize this condition did not give any improvement of the crystal quality or the success rate.
Results and discussion

As mentioned above, only 10% of the obtained crystals were of sufficient quality for data collection, i.e. did not show too high anisotropy and/or mosaic spread. Finally, a data set could be collected to a resolution of 3.3 Å. Autoindexing indicated that the crystals belonged to the monoclinic space group C2, with 8-10 monomers in the asymmetric unit. The quaternary structure of native DmβAS is undetermined, native gel electrophoresis experiments show a mixture of oligomeric states. We also performed an analytical gel filtration, which suggested an octamer or even higher oligomeric assemblies.

A calculated self rotation function showed one peak at 78.5% of the origin peak height and (θ=120.0°, ϕ=180.0°), and also several additional peaks at 26-46% of the origin peak height, indicating that a number of non-crystallographic two folds axes are present in the asymmetric unit.

Several attempts to determine the structure of DmβAS by molecular replacement were made using four different programs. Several structure models were used, e.g. DCase (24% amino acid identity) and the hypothetical protein PH0642 (28% amino acid identity). All models were manipulated in different ways, including sequence adjustments and truncations. We also tried different oligomeric states, e.g. monomers, dimers and tetramers, but no clear molecular replacement solution could be identified at first.

Conclusion

After several attempts and screens, we succeeded to produce crystals from native DmβAS. Unfortunately, these crystals diffracted poorly and decayed rapidly in the X-ray beam. Finally, a data set could be collected to 3.3 Å in the monoclinic space group C2. Unfortunately, phasing by molecular replacement proved very difficult. For this reason we initiated production of selenomethionine substituted DmβAS for structure determination by MAD phasing.
Structure determination of DmβAS

Structure determination and quality of the model

A molecular replacement solution could finally be obtained using a truncated and sequence adjusted dimeric model of the protein PH0642 and the program PHASER. Four dimers were placed in the asymmetric unit with Z-scores of 6.7, 14.2, 17.8 and 5.8. After initial refinement cycles it became apparent that the fourth dimer was incorrectly placed, this could however later be located manually by careful inspection of the electron density maps and application of non-crystallographic symmetry relations. The model building using the first data set (nat1) was at its final stages when we were able to obtain a new crystal form using nanodrop crystallization approaches but otherwise similar conditions. An X-ray data set could be collected with improved quality and resolution (2.8 Å, nat2). The crystal belonged to space group P2₁2₁2₂.

The structure was determined by molecular replacement using a monomer of the nat1 model. Three monomers were found in a single run of the program MOLREP and a fourth was correctly placed after an additional run for which the first three monomers were fixed.

In the crystals of space group C2 tight dimeric units assemble into a homooctamer. The same overall structure and dimer organization is observed also for the four subunits present in the asymmetric unit of the P2₁2₁2₂ crystal form (nat2), which form half an octamer. A full octamer can be assembled by application of crystallographic symmetry. The final models refined to R-values of 22.6/21.5% and R<sub>free</sub> = 28.2/25.5%, and the Ramachandran plots show good stereochemistry for both models.

The electron density is continuous and well defined in both space groups. Exceptions regard the “corner subunits” (nat1: chain A and H, nat2: chain A), in which electron density is missing for three loop regions, which are exposed to bulk solvent only in the corner subunits.

Subunit structure

The DmβAS monomer exhibits the characteristic αββα four-layer sandwich fold characteristic for the nitrilase superfamily (fig.16). Two β-
hairpins shield the active site from the surrounding solvent and take part in the dimer-dimer interactions within the octamer (fig. front page).

Figure 16. The monomer of DmβAS, colored by its secondary structure, β-strands in light gray and α-helices in black. The catalytic triad is shown as sticks and colored in dark gray.

Subunit interactions

Two different experimental methods indicate the presence of several oligomeric states for DmβAS. The physiologically relevant quaternary structure of DmβAS is still unknown. The crystal structure of DmβAS reveals a homo-octamer in the shape of an almost complete helical turn. Two monomers form a tightly intertwined dimer with an eight-layered αβα-αβα sandwich fold. About 4000 Å² of the monomer accessible surface area are buried in the dimer interface which is dominated by hydrophobic interactions (X in fig. 17a). There is a four-helical bundle-like protrusion formed by the two N-terminal helices from each subunit, and
Figure 17. a) The tetramer of DmβAS from the front with the three most
important interfaces labeled X, Y and Z. The first dimer is colored in yellow and blue, the second is in mangenta and green. b) DmβAS tetramer viewed from the back with the C-terminal embracing the other monomer in the dimer. c) The superimposed active sites of DmβAS (stick models of residues with carbon atoms in magenta) and of the Agrobacterium sp. DCase double mutant C171A/V236A (carbon atoms in green) in complex with its substrate N-carbamyl-D-valine (carbon atoms in yellow). Hydrogen-bonding interactions between DCase and its substrate are indicated by dotted lines. Model of a higher oligomeric state (24-mer) of DmβAS in surface representation. (d): Side view of a possible helix formation modeled by assembling several octamers. To generate complete interfaces at the front of the helix, the disordered loops were modeled and included in subunit H of the bottom octamer (green), in subunits A and H of the central octamer (blue) and in subunit A of the octamer at the top (magenta). (e): Top view of the helix. The small protrusions on the outer surface are formed by the interacting N-terminal helices of each dimer couple.

the C-terminal regions are closely packed against the partner subunit (fig. 17b). All of the dimer-dimer interactions within the octamer are equivalent, with ~1210 Å² of the monomer surface buried in the interface between the diagonally connected subunits (Y), and ~730 Å² buried between direct neighbors (Z). The three residue stretches for which no electron density was observed in the corner subunits are in all other subunits involved in the diagonal interaction.

**Comparison with other nitrilase-like enzyme structures**

Several structures of enzymes that belong to different branches of the nitrilase superfamily were identified after a structure similarity search with DmβAS (table 4) using the Protein Structure Comparison service SSM. The structural fingerprint of these proteins is the αββα-fold of the monomer that is extended to an eight layer αββα–αββα sandwich fold upon dimerization, and a conserved Cys-Glu-Lys catalytic triad. When the DmβAS crystal structure is compared with its closest relative DCase it has an extension of 67 amino acids at the N-terminus and of 13 amino acids at the C-terminus. The N-terminal extension is unique for DmβAS and has not been observed in any other known structure from the nitrilase superfamily. This N-terminal extension prevents DmβAS from forming certain oligomeric arrangements otherwise observed in the superfamily. For instance, severe steric clashes are observed involving the
N-terminal residues when a subunit of DmβAS is superimposed onto one of the subunits in the tetramer of DCase or in the hexamer of an amidase. In contrast, the C-terminal extension is observed in other structures and does not cause any steric clashes.

**The active site**

Cysteine 234 has been identified as the catalytic nucleophile in DmβAS based on sequence conservation as well as functional and structural similarities to enzymes where the important role of this cysteine has been elucidated by site directed mutagenesis (Hung et al., 2007, Chen et al., 2003). The active site is accessible to bulk solvent through a narrow channel leading to Cys234. In the corner subunits this channel is widened to a shallow groove because of the disorder of three loops.

Residual electron density was found in the active sites near Cys234 for all monomers in nat1 and nat2, except for the corner subunits. Because of the moderate resolution we can only speculate about the nature of the bound molecule. One possibility is the substrate NCβA, which is present in both the nat1 and nat2 crystallization setups and may not have been turned over because of the low pH, 4.2, of the condition. Another possibility is the product β-alanine, which could have been generated from the substrate NCβA, but we cannot exclude that something else from the crystallization condition could be associated with the electron density.

Residues involved in binding the carbamyl and carboxyl groups of the carbamylated D-amino acids in DCase have been identified by structural analysis of several complexes between inactive enzyme and diverse substrates. Because of the conservation of these functional groups between NCβA/N-carbamyl-β-aminoisobutyrate and the DCase substrates, substrate binding in DmβAS may be achieved in a similar way as in DCase. A superposition of the active site of DmβAS and the inactive mutant of DCase C171A in complex with the substrate N-carbamyl-D-valine was performed, and the residues possibly involved in substrate binding were compared (fig.17c). The catalytic triad Cys-Glu-Lys is structurally well conserved between the two enzymes. The carboxyl group of the substrate is in DCase coordinated by several hydrogen bonds to the side chains of Asn172, Arg174, Arg175 and Thr197. The two arginines were found to be essential for substrate binding by site directed mutagenesis. These amino acids correspond to Arg237 and His238 in DmβAS. The substrate NCβA was modeled into the active site of
DmβAS. This model places the carboxyl group of the substrate in hydrogen bonding distance to Arg237 and His238. When the active site pockets of DmβAS and DCase are compared, the active site of DmβAS is narrower, as expected from the size of the different substrates used.

**Figure 18.** Proposed reaction mechanism of N-carbamyl-β-alanine to β-alanine performed by DmβAS.
Catalytic mechanism

This mechanism has been proposed earlier for the DCase whose substrate also contains a N-carbamyl group and has similar chemical properties as NCβA (for the proposed mechanism see fig. 18 (Nakai et al., 2000)). The carboxyl group of Glu120 serves as a general base catalyst and abstracts a proton from the thiol group of Cys234. The activated nucleophile attacks the amide carbon of NCβA to form a tetrahedral intermediate, which is stabilized by the amino group of Lys197. The intermediate subsequently collapses to form an acyl-enzyme complex and releases ammonia, which may have received a proton from Glu120. Deacylation is accomplished by nucleophilic attack by a water molecule that replaces ammonia. This water molecule is activated by Glu120 via a general-base activation mechanism. The released intermediate is most likely spontaneously decarboxylated to β-alanine.

Implications for allosteric regulation and formation of higher oligomers

DmβAS exists in different oligomeric states, of which the smallest is most likely a dimer. The helical turn-like structure of DmβAS suggests that higher oligomeric states could easily be achieved by adding dimers to the ends of the octamer, which would result in a left-handed spiral arrangement. To see if this arrangement is at all possible, a helical model composed of 12 dimers was created (fig. 17d and e). Model analysis using the Pisa server revealed a set of new interactions compared to those found in the octamer.

The N-terminal extension that for DmβAS prevents an oligomerization as observed for DCase is also found in e.g. human, rat and plant βAS (fig. 19). It can therefore be assumed that the enzyme assembles in a similar way as DmβAS. Rat liver βAS has been shown to be allosterically regulated. It adopts different oligomeric states dependent on the presence of substrate or product. A comparable behavior could not be detected for DmβAS in preliminary gel filtration experiments.

Three enzymes from the nitrilase superfamily, the cyanide-degrading enzymes from Pseudomonas stutzeri and Bacillus pumilus and the nitrilase from Rhodococcus rhodochrous J1, have been shown to form helical assemblies by electron microscopy studies (Sewell et al., 2005, Thuku et al., 2007). These assemblies have a diameter of 95-130 Å and a pitch of 77-95 Å. The diameter of the modeled DmβAS helix is ~120 Å and
corresponds well to that of nitrilase helices, while the pitch is significantly smaller, 65-70 Å. If the helical state of DmβAS exists \textit{in vivo}, it would be more tightly packed along the long axis of the helix, resulting in more extensive contacts between the subunits.

\textbf{Figure 19.} Amino acid sequence alignment of DmβAS and homologous proteins: row 1, DmβAS; row 2, human βAS; row 3, rat βAS; row 4: \textit{Agrobacterium sp.} DCase. The sequences were aligned using the program MULTALIN. For DmβAS, the secondary structure elements as analyzed by DSSP are given, with arrows representing β-strands, and spirals representing helices. Black background shading indicates amino acid conservation in all five sequences, bold letters and boxes are used when a residue is conserved in some of the sequences and conservatively exchanged in the remaining ones.
Conclusion

Here we report the first crystal structure of a βAS from a higher eukaryote belonging to another subfamily than the earlier characterized yeast enzyme. For DmβAS, the same quaternary structure was found in two different crystal forms. The structure is a homooctamer associated from tightly packed dimers. The active site pocket contains the catalytic triad that is strictly conserved within the nitrilase superfamily. A reaction mechanism is proposed that is highly similar to that used by the closest functional neighbor DCase.

Additional unpublished results

To determine which fold, active site architecture and catalytic mechanism is better suitable for performing the βAS reaction we compared the kinetic parameters of βAS from both subfamilies. Furthermore, we tested whether yeast βAS can bind and be inhibited by peptides, which would give further evidence for its phylogenetic relationship with bacterial dizinc-dependent exopeptidases.

βAS kinetic parameters differ significantly

A radioactive enzyme assay was used (Lundgren et al., 2007) to determine the kinetic parameters of DmβAS, A. thaliana βAS (AtβAS) and SkβAS. All three enzymes exhibit reasonable reaction velocities with \( V_{\text{max}} \) values between 0.4 – 7.1 U/mg, while the \( K_\text{M} \) values differ significantly. Both DmβAS and AtβAS had reasonable \( K_\text{M} \) values of 24.5±2.4 \( \mu \text{M} \) and 6±1 \( \mu \text{M} \), respectively, while for SkβAS the \( K_\text{M} \) is about 1000 times higher, 50-70 mM. Hence, the conservative βASs from D. melanogaster and A. thaliana are more effective in the conversion of NCβA to β-alanine than SkβAS, which most likely evolved via recruitment and alteration of the structural scaffold of exopeptidases.

SkβAS is inhibited by di- and tripeptides

The three different βASs were compared regarding their inhibition properties. Several compounds that are chemically similar to the substrate or the product were tested. A couple of oligo-peptides were also tested
because of the structural similarities between $Sk\beta$AS and exopeptidases. The experiments were performed at substrate and product concentrations that coincide with the $K_M$ values observed for the different enzymes. Three compounds in particular were identified as inhibitors of Dm$\beta$AS and At$\beta$AS, with each decreasing $V_{\text{max}}$ to below 80% of maximum activity under standard reaction conditions (pH 7.0 and 25°C) and at a concentration of 100 $\mu$M of the inhibitor and 20 $\mu$M of the substrate. The identified compounds were isobutyric acid, glutaric acid (resembling the product $\beta$-alanine) and carbamyl phosphate (resembling one of the possible reaction intermediates).

$Sk\beta$AS is inhibited by $\beta$-alanine, isobutyric acid, 2-amino isobutyric acid, N-carbamyl amino isobutyric acid and carbamyl phosphate with a decrease of $V_{\text{max}}$ between 30-80%, at a substrate concentration of 25 mM and an inhibitor concentration of 50 mM. Also oligopeptides carrying a glycyl residue at the N-terminus inhibited the yeast enzyme. The most efficient inhibitors were Gly-Gly, Gly-His, Gly-Leu, Gly-Phe and Gly-Ser, each of which decreased $V_{\text{max}}$ to <25% of that of the uninhibited reaction.

**Inhibitor binding**

Crystals of wild type $Sk\beta$AS were grown in the presence of Gly-Gly, Gly-His, Gly-Leu, Gly-Phe and Gly-Ser and complete datasets were collected for each kind of co-crystal. After a round of refinement with ligand-free $Sk\beta$AS, ligand-associated positive electron density peaks were observed only for Gly-Gly and Gly-Ser. A dataset was also collected of the $Sk\beta$AS mutant R322A in complex with Gly-Gly. The inhibitors are bound in the substrate binding site in both subunits in the $Sk\beta$AS dimer, making even more extensive contacts to the protein than previously observed for the substrate, NC$\beta$A. (fig. 20) The same pattern of a not fully occupied di-zinc center (discussed in paper II) is also observed in the WT and mutant structure of the inhibitor complexes.

The main difference between the binding of the substrate and the inhibitor are the partial occupation of the space available for zinc2 and the interactions with zinc1. The amino group of the N-terminal glycine of the inhibitor coordinates to zinc1 at one of its four coordination positions, the other three are occupied by D125, H114 and H226. In the E159A_NC$\beta$A structure, a water molecule is present at this position to be activated by E159 for the nucleophilic attack on the substrate (Lundgren *et al.*, 2007). The occupation of the substrate binding site by the inhibitor, its
coordination to zinc1 and the displacement of zinc2 and the catalytic water contribute to the inhibition of the enzyme.

Figure 20. The active site of SkβAS with bound inhibitors. Hydrogen bonds to the zinc ion and ligands are indicated by dotted lines. The zinc is colored black. A) WT-SkβAS in complex with Gly-Gly. B) SkβAS mutant R322A in complex with Gly-Gly. C) WT-SkβAS in complex with Gly-Ser.

Conclusion

In this study we show that the βASs from higher eukaryotes have higher affinity for the substrate NCβA than SkβAS does. We also observe that short peptides inhibit SkβAS and engage in even more extensive interactions to the protein than the substrate NCβA. Two possible reasons
why, after the loss of the original βAS ancestor gene in the yeast lineage, a peptidase could be recruited to perform the βAS reaction in *S. kluyveri* are:

a) Both enzymes catalyze the hydrolysis of a carbon-nitrogen bond.
b) The substrates of both enzymes have a similar distribution of functional groups.

When the two βAS structures studied in this thesis are compared with each other, there are no clear signs that would indicate that these two enzymes perform the same reaction. *First*, both the enzymes have an α/β fold, but the overall fold is significantly different. DmβAS is a one-domain enzyme while SkβAS has two domains, one catalytic domain and a dimerization domain. *Second*, it seems like both enzymes need to be assembled into higher oligomeric states to be able to perform the reaction. This has been shown to be true for SkβAS as two of the substrate anchoring residues belong to the neighboring subunit. It may also be true for DmβAS, since it was observed that if one of the three important subunit interfaces is missing, the three loops closing the active site are flexible and could inhibit the reaction. *Third*, the native quaternary structures of the two enzymes are different to a large extent, even though it seems like the smallest oligomeric states for the two enzymes are dimers. DmβAS can form at least homooctamers, while the yeast enzyme is a dimer and cannot form larger assemblies. *Fourth*, the reaction mechanisms for the two enzymes are very different. SkβAS uses a metal mediated reaction mechanism with an activated hydroxyl as a nucleophile, while the reaction in DmβAS uses a Cysteine residue as the nucleophile.

Based on the data presented here, it seems most likely that the SkβAS was originally an exopeptidase that was recruited to convert NCβA to β-alanine. This reaction is, in most eukaryotes, performed by a βAS encoded by the seemingly unrelated conventional *pyd3* gene and thus represents an example of convergent evolution.
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During my first years as a student I had the best support that one can hope for from my lab-group. I am so happy that we have stayed in touch after we finished our studies. I will always remember things like throwing oranges at restaurants or preparing dinners together. Thank you for your friendship Ylva, Fredrik, Benita, Karin, Max, Lotta, Malin and Cissi.

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