β-Alanine synthase (βAS, EC 3.5.1.6, also called β-ureido-propionase) catalyzes the third and final step of the reductive pyrimidine catabolic pathway in which propionase) catalyzes the third and final step of the reductive pyrimidine catabolic pathway, which is responsible for the breakdown of uracil and thymine in higher organisms. The fold of the homodimeric enzyme from the yeast Saccharomyces kluveri identifies it as a member of the AcyI/M20 family of metallopeptidases. Its subunit consists of a catalytic domain harboring a di-zinc center and a smaller dimerization domain. The present site-directed mutagenesis studies identify Glu[159] and Arg[222] as crucial for catalysis and His[262] and His[397] as functionally important but not essential. We determined the crystal structures of wild-type β-alanine synthase in complex with the reaction product β-alanine, and of the mutant E159A with the substrate N-carbamyl-β-alanine, revealing the closed state of a dimeric AcyI/M20 metallopeptidase-like enzyme. Subunit closure is achieved by a ~30° rigid body domain rotation, which completes the active site by integration of substrate binding residues that belong to the dimerization domain of the same or the partner subunit. Substrate binding is achieved via a salt bridge, a number of hydrogen bonds, and coordination to one of the zinc ions of the di-metal center.

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Eukaryotic βASs have been purified and characterized from a number of sources (11–15). Based on the comparison of primary structures and phylogenetic analyses they can be assigned to two subfamilies (15). The majority of the eukaryotic βASs belong to one subfamily. βAS from the yeast Saccharomyces kluveri (SβAS, encoded by PYD3) shows negligible sequence similarity to this group but is related to bacterial N-carbamyl-L-amino acid amidohydrolases, hence forming a separate subfamily. Thus far, it is the only βAS whose crystal structure has been determined (16). The subunit of the homodimeric enzyme consists of a catalytic and a dimerization domain. The active site is located in the domain interface and contains a di-zinc center exclusively bound by residues of the catalytic domain. Its coordination is characteristic for the metallopeptidase H clan of zinc peptidases (17). The overall structure identifies SβAS as a member of the aminoacylase-1/metallopeptidase 20 family (AcyI/M20) (18), which comprises proteins with diverse metabolic functions. Some of these proteins have therapeutic significance, such as human serum carnosinase, a promising drug target in the treatment of diabetes and homocarnosinosis (19), and carboxypeptidase G2 from Pseudomonas sp. strain R5-16 that is under development as a rescue agent in cases of methotrexate overdoses (20).

Members of the AcyI/M20-family exist as either monomers or homodimers. In each of the crystal structures determined for homodimeric members the subunits display an extended open conformation (16, 21, 22). For SβAS, AcyI, and others, it has been suggested that a few residues from the dimerization domain participate in substrate binding and catalysis (16, 23).
Because they do not approach the active site close enough in the crystal structures, it was proposed that conformational changes in form of domain movements are part of the catalytic cycle. The monomeric PepV from *Lactobacillus delbrueckii* is thus far the only member of the Acyl/M20 family for which the structure of a closed complex has been determined (24).

In this study we report the crystal structures of a SkβAS mutant complex with NCβA and of the wild-type enzyme in complex with β-alanine, which for the first time show the enzyme in the closed conformation and reveal the mode of substrate and product binding. Furthermore, we studied the role of specific active site residues for catalysis and substrate binding by site-directed mutagenesis. The crystal structures of some of the mutant enzymes were determined to analyze the structural effects of the amino acid exchanges.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis and Protein Purification**—Site-directed mutagenesis of SkβAS was performed on the plasmid P491 carrying the *S. kluyveri* PYD3 gene followed by a fragment encoding the C-terminal His8 tag (15). The QuikChange XL Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) was used to introduce the desired mutations. Forward versions of the mutagenic primers are listed in supplemental Table S1, with the mutation site underlined. The sequences of the mutant SkβAS genes in the resulting transfer vectors were confirmed by DNA sequencing.

Wild-type and mutant SkβAS were recombinantly expressed and purified as described by Gojković et al. (15), with only minor modifications of the purification protocol. Protein concentration was determined by the method of Bradford (25) using bovine serum albumin as a standard (for crystallizations) or spectrophotometrically at 280 nm using the molar extinction coefficient of 62340 M⁻¹ cm⁻¹ calculated with the ProtParam tool of the ExPASy proteomics server (for kinetic measurements).

**Enzyme Kinetics**—The activity of wild-type and mutant SkβAS was determined in a radiochemical assay detecting the ¹⁴CO₂ released from the substrate, basically following the procedure described by van Kuilenburg et al. (26). The standard assay mixture contained 0.1 M sodium phosphate, pH 7.0, 10% (v/v) glycerol, and 1 mM ZnCl₂; NCβA concentrations were varied in the range of 1–100 μM. For the determination of the pH dependence of the SkβAS activity an NCβA concentration of 80 μM was used. The reaction was allowed to proceed for 5–15 min at 25 °C. For each substrate concentration and enzyme sample, the reaction and measurement were performed three times. The data were fitted using the Michaelis-Menten equation. One unit of SkβAS catalyzes the hydrolysis of 1 μmol of NCβA/min at 25 °C.

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![Scheme 1. The reductive pyrimidine catabolic pathway.](image)

**Thermal Shift Assay**—The thermal shift assays for SkβAS-WT and SkβAS-H226E and the analysis of the thermal shift data were performed as described by Ericsson et al. (27). The thermal stability of both proteins was tested using the same buffer as for the radiochemical assay (100 mM sodium phosphate, pH 7.0, 10% (v/v) glycerol, 1 mM ZnCl₂) containing different concentrations of NaCl (0, 50, 100, 200 mM), in the absence or presence of 75 mM NCβA or β-alanine, respectively. This substrate/product concentration corresponds to the one used for the co-crystallizations resulting in SkβAS-ligand complexes. The protein concentration was 0.4 mg/ml.

**Crystallization and Data Collection**—To obtain the crystals for the datasets R322A and E159A³ (and of the mutant E159D) the original crystallization conditions (28) were employed with only minor changes. For instance, for R322A, the original crystallization buffer was replaced by 50 mM sodium citrate, pH 6.0 mixed with 50 mM Na-Hepes pH 7.0. Addition of potential ligands (R322A: 20 mM NCβA; E159A: 50 mM Gly-Gly-Gly; corresponding to active site/ligand molar ratios of 1:250 and 1:625, respectively) to the protein solution did not influence the crystallization behavior, and led only for R322A to appearance of electron density peaks confirming ligand binding. The drops consisted of 1.5 μl of protein solution and 1.5 μl of reservoir solution. The crystals appear after a 2–4-week equilibration at 20 °C and do not require cryoprotection.

The conditions producing crystals for datasets E159A_NCβA and WT_βAla were identified in a subsequent round of sparse matrix and grid screening using commercially available formulations and nanodrop technology. Spherulites appeared almost immediately in some conditions of the polyethylene glycol (PEG) 6000/LiCl grid screen after set-up of the 96-well sitting drop plates at 20 °C. These conditions were further optimized. The best crystals were obtained using vapor diffusion against 1 ml of reservoir solution containing 22–25% PEG 6000, 50 mM Tris, pH 8.5, 50 mM Bicine, pH 9.0, and 1 mM LiCl. The hanging drops consisted of 1 μl of protein solution (4–4.5 mg/ml SkβAS-E159A or ·WT, 10 mM dithiothreitol, 75 mM NCβA or β-alanine, corresponding to an active site/ligand molar ratio of about 1:900) and 1 μl of reservoir solution, and were immediately after setup streak-seeded with seeds from previously obtained spherulites or crystals. Before data collection, the crystals were transferred into a 2-μl drop of cryo solution for about 5 s. The solution consisted of 1.3 μl of the respective reservoir solution, 0.6 μl glycerol (99%) and 0.1 μl of the respective ligand solution, ensuring the same final ligand concentration as used for the co-crystallization.

All crystals were flash-frozen in a nitrogen gas stream. X-ray diffraction data were collected at 100 K at diverse beam lines of the ESRF (Grenoble, France) (Table 1). The data sets WT_βAla and E159A_NCβA were processed and scaled with XDS (29), the others were processed with MOSFLM and scaled with

³ Italics refer to datasets or crystal structures, normal font refer to mutant proteins or mutation sites.
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SCALA of the CCP4i suite of programs (30). Table 1 gives details of the data collection statistics.

Structure Determination and Analysis—The structures were solved by molecular replacement using the program MOLREP (31) with the coordinates of ligand-free SkβAS (PDB-ID 1r43) as a search model. First, the positions of the dimer(s) of the dimerization domain were determined and fixed, while in the second step the coordinates of a single catalytic domain were used as search model. For data collected from crystals of the mutant E159D, the orientation and position of one catalytic domain per homodimer could not be determined, most likely because of too large variations in the interdomain angle between subunits related by crystallographic symmetry. Because the refinement of a model missing this domain resulted in a relatively poorly defined electron density map and non-satisfactory refinement statistics, these data were excluded from further analysis.

Cycles of model building using WinCOOT (32, 33) and O (34) were alternated with TLS and restrained structure-factor refinement in REFMAC5 (35, 36) until crystallographic R-factor and Rfree converged. TLS groups were defined based on analyses using the TLSMD server (37). Tight main chain and medium side chain NCS restraints were used throughout the refinement and only released for parts of the polypeptide chains, which showed clear structural differences between the subunits. NCβA or β-alanine were fitted into correspondingly shaped electron densities observed for R322A, E159A_NCβA, and WT_/βAla, respectively. Water molecules were added using ARP/WARP implemented in the CCP4i suite of programs (30) or manually in WinCOOT, and alternative conformations of amino acids were introduced and refined with appropriately split occupancies when the electron density maps indicated their presence. For E159A_NCβA and E159A, the electron density observed for one of the zinc ions (zinc 2) is too weak to represent a fully occupied zinc site. Because the distances to surrounding residues are shorter than the length of a hydrogen bond (<2.4 Å), replacement of the zinc by a water molecule could be excluded. Therefore, the refinement was carried out with the occupancy for zinc 2 set to 0.3. The composition and quality of the final models and further refinement statistics are given in Table 1.

The model quality was determined using the program PROCHECK (38). Structural alignments were achieved with the programs TOP (39) and LSQKAB (40). Crystal, ligand, and monomer-monomer contacts were analyzed with CONTACT of the CCP4 suite of programs (30) and the Protein-Protein Interaction server, respectively. Domain dynamics were analyzed using the DynDom server (41), and cavities were calculated using the CASTp server (42). Figs. 1, 2, b and c, and 3 and supplemental Fig. S2 were generated using PyMOL (43). The crystallographic data and structures have been deposited in the Protein Data Bank, with the accession codes 2v8v (R322A), 2v8d (E159A), 2v8h (E159A_NCβA), and 2v8g (WT_/βAla).

RESULTS AND DISCUSSION

Site-directed Mutagenesis, Purification, and Crystallization of SkβAS Mutants—Because no crystal structures for SkβAS complexes mimicking the substrate-bound state could thus far be obtained, residues putatively involved in ligand binding and catalysis were identified by comparisons with related enzymes and modeling studies (16). Among them are the glutamate residue (Glu199) that is strictly conserved in Acyl/M20 family members and suggested to act as the catalytic base, His397, Gln229, and Gly396 putatively binding the substrate carbamyl group, as well as Arg322, His762, and Asn309 implicated in the binding of the carboxyl group.

To test our hypotheses we produced six SkβAS mutants with single amino acid exchanges. Two of the three putative carboxyl group anchors were mutated to alanine (R322A, H262A), and Glu199 was exchanged with aspartate (E159D) or alanine (E159A). The metal-coordinating His226 was replaced by glutamate (H226E) to mimic the di-zinc center coordination observed in most members of the Acyl/M20-family, while His397 was mutated to an amino acid with an uncharged side chain of similar length (H397N).

Expression of all mutant genes resulted in similar amounts of soluble protein as expression of the native gene. All mutants could be purified to a similar degree of homogeneity (supplemental Fig. S1). Using the original crystallization conditions diffraction-quality crystals were obtained for SkβAS-R322A, -E159A, and -E159D. The crystals of SkβAS-R322A belong to space group P1, with four subunits per asymmetric unit. SkβAS-E159A and -E159D crystallize in space group P21. The unit cell dimensions correspond to those previously observed for the selenomethionine-substituted protein, placing two monomers in the asymmetric unit. Co-crystallizations with NCβA, β-alanine, or potential ligands did not result in ligand binding to the active site.

Another round of crystallization screens led to the identification of a novel condition at higher pH (8.75). Optimization of the condition yielded diffraction-quality crystals for SkβAS-WT and four of the mutants. All belong to space group P21, with consistent unit cell dimensions (Table 1) that do not correspond to those of crystals grown under the original condition.

Because SkβAS-H226E failed to crystallize under both conditions, a thermal shift (Thermofluor) assay was performed to compare the thermal stabilities of this mutant and the native enzyme (supplemental Fig. S1b). It revealed that the low crystallization propensity of SkβAS-H226E is not caused by structural instability or incomplete folding, because the melting temperatures of mutant and wild-type enzyme are basically identical and the unfolding curves exhibit the same shape at all tested conditions.

Overall Structure—As described previously, the subunit of SkβAS is composed of two domains (16). The smaller domain (residues 247–365) consists of four β-strands (one of which is split) packed against two long α-helices and mediates most of the subunit contacts within the homodimer. This domain is inserted into the larger catalytic domain (residues 1–246, 366–455), which has a mixed three layer α/β/α-sandwich topology. The average root-mean-square distances (r.m.s.d.) for separate superposition of all catalytic and dimerization domains of the structures described here are ~0.44 and 0.33 Å, respectively. There are only two noteworthy deviations between backbone coordinates. The deletion of the Arg322 side chain induces in
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TABLE 1

Data collection and refinement statistics

|                | R322A | E159A | WT βAla | E159A NCβA |
|----------------|-------|-------|---------|------------|
| Data collection statistics |       |       |         |            |
| Wavelength (Å)    | 0.874 | 0.939 | 0.979   | 0.931      |
| Temperature (K)   | 100   | 100   | 100     | 100        |
| X-ray source      | ID23-2 ESRF | ID14-4 ESRF | ID23-1 ESRF | ID14-3 ESRF |
| Resolution (Å)*   | 45.0-2.9 (3.06-2.90) | 50.0-2.3 (2.42-2.30) | 20.0-2.5 (2.60-2.50) | 20.0-2.0 (2.10-2.00) |
| Space group       | P1    | P21   | P21     | P21        |
| Unit cell         | a b c (Å) | 76.9 84.4 104.0 | 61.1 77.1 108.4 | 50.1 217.3 81.6 | 49.8 218.3 81.6 |
| α β γ (°)         | 67.3 68.1 63.4 | 90.0 97.1 90.0 | 90.0 91.9 90.0 | 90.0 92.2 90.0 |
| Molecules/AU      | 4     | 2     | 4       | 4          |
| Reflection no.    | Overall | 93844 (13600) | 38138 (15330) | 215070 (23499) | 481766 (65677) |
| Unique            | 44094 (6535) | 43156 (5719) | 59732 (6593) | 116114 (15708) |
| R Factor (%)       | 19.4  | 23.0  | 18.9    | 17.8       |
| Rfree (%)          | 24.6  | 27.6  | 23.1    | 20.8       |
| no/13-factor (Å²) | 13363/39.1 | 6685/35.9 | 13413/27.8 | 13585/26.9 |
| Protein atoms a    | 8/34.2 | 4/30.5 | 8/48.2 | 8/40.9 |
| Zinc ions a        | 4/34.1 | 63/21.5 | 210/22.7 | 956/33.2 |
| Water molecules a  | 27/61.9 | - | 12/39.6 | 36/34.9 |
| Wilson-B (Å²)      | 46.5  | 48.8  | 33.2    | 28.4       |
| Refinement statistics | Resolution | 45.0-2.9 | 45.2-2.3 | 19.8-2.5 | 19.8-2.0 |
| Reflection no.     | Working set | 41837 | 40838 | 56799 | 110321 |
| Test set           | 2231 | 2195 | 2931 | 5792 |
| R-factor (%)       | 19.4 | 23.0 | 18.9 | 17.8 |
| Rfree (%)          | 24.6 | 27.6 | 23.1 | 20.8 |
| Ramachandran plot, residues in | Bond distance (Å) | 0.011 | 0.010 | 0.010 | 0.009 |
|                      | Bond angle (°) | 1.37 | 1.13 | 1.18 | 1.10 |
| Properties         | Crystallization pH | 6.0 | 6.0 | 8.75 | 8.75 |
|                    | Open/closed | Open | Open | Closed | Closed |
|                    | Zinc centre | Complete | Incomplete | Complete | Incomplete |

Note:

* Values for the highest resolution shell are given in parentheses.

Additional electron density best fitted by bicine molecules is observed in both closed state structures in an area that can be regarded as the neck of the hinge region, suggesting that the

one subunit per R322A dimer a shift of two amino acid stretches at the tip of the dimerization domain up to 1.6-Å closer toward the active sites. Residues 259–273 probably simply follow a preceding shift of stretch 303–313 toward the gap left by the deleted side chain, as they are connected by several backbone hydrogen bonds. Why this movement occurs in only one monomer per dimer remains unclear; crystal packing interactions do not appear to be the cause. Furthermore, as a consequence of ligand binding two loop regions from the catalytic domain (161–167, 186–195) located at the entrance to the active site are shifted up to 1.8 Å closer toward it in E159A NCβA and WT βAla when compared with their position in the ligand-free enzyme structures.

Analysis of the subunit as a whole reveals major differences between the diverse mutants or complexes. E159A NCβA and WT βAla represent the closed conformational state of SkβAS (Fig. 1a), while the old crystallization conditions continued to produce open states for R322A and E159A (Fig. 1b). The eight closed subunits from both complexes superimpose very well with an average r.m.s.d. of 0.12 Å. In contrast, as the angle between the dimerization and catalytic domain is not fixed, the open forms do not adopt exactly the same overall structure (Fig. 1c), reflecting a higher degree of flexibility associated with this enzyme state. SkβAS-R322A adopts the most open configuration. The electron density for all four chains per asymmetric unit is equally well defined, suggesting that the observed open state is sufficiently stabilized by the molecular packing in the triclinic lattice. In contrast, in the crystals belonging to the monoclinic spacegroup P21 that resulted in SkβAS-E159A and previously reported open-state structures, one of the catalytic domains per dimer has freedom to adopt different orientations, as indicated by its weaker definition in electron density and larger B-values. In fact, for E159D the position of one of the catalytic units could not be determined. Analysis of dissolved crystals by SDS-polyacylamide gel electrophoresis and the presence of strong peaks of electron density at the approximate position of the di-zinc center of the missing domain do however indicate that no proteolytic cleavage has occurred.

For the open states, there is no electron density observed for the N-terminal 17–18 residues and the C-terminal His455. In the closed structures, the mobility of the N-terminal sequence is even more extended up to Leu28, while the C terminus becomes more ordered so that three amino acids of the linker sequence preceding the His tag are now visible in the electron density map, forming an additional turn of the C-terminal helix.

Additional electron density best fitted by bicine molecules is observed in both closed state structures in an area that can be regarded as the neck of the hinge region, suggesting that the
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The Closed State—Closed states have been modeled for several dimeric Acyl/M20 family members (44–46), but SkβAS is the first enzyme for which the closed conformation could be crystallized and characterized based on experimental data. The transition from the open state (K322A) to the closed state of SkβAS (E159A_NCβA, WT βA) involves a rotation of the catalytic domain relative to the dimerization domain by ~30° (Fig. 1, a–c). This is achieved by changes in Φ and Ψ angles of residues 245–247 and 363–366 (up to 28°), which connect both domains and serve as a hinge region. In the closed state the side chains of Asp192 located at the tip of the large loops protruding from the catalytic domains toward each other are engaging in van der Waals interactions, while their Cα atoms are separated by a distance of ~32 Å in the open state. The closed state is characterized by an increased monomer-monomer as well as interdomain surface (Fig. 1, d and e). The area buried between the catalytic domain of one particular subunit and the rest of the dimer is about two times larger in the closed state (~2500 Å²) than in the open state structures (~1200 Å²). Several new strong interactions are formed in the domain interface involving a large number of residues (Fig. 1e), of which some are important for substrate binding and catalysis. The side chain of Asn309, for instance, becomes hydrogen-bonded to the zinc ligand Glu160 and Gln118, which in turn forms an additional hydrogen bond with Ser307. The side chain of Arg322 interacts with Gly394, while the backbone of Gln229 becomes hydrogen-bonded to Thr265. His262 is now in van-der-Waals distance to Ser420 and the zinc ligand His421. Tyr246, a residues found in the generously allowed region of the Ramachandran plot, is also involved in domain closure contacts. As previously predicted, the most important consequence of the global conformational change is the movement of Arg322 from the dimerization domain of the same subunit, and Asn309 and His262 from the corresponding domain of the partner subunit into the active site, where they can fulfill their crucial functions in substrate binding and catalysis.

Metal Binding Properties—The active site of SkβAS contains a di-nuclear zinc center, which is accessible from the cleft between the catalytic and the dimerization domain. Its coordination differs in one respect from that of most Acyl/M20 family members: the zinc ion commonly termed metal 1 has two histidine ligands (His114, His226) in addition to the bridging aspartate (Asp125), instead of one histidine and one acidic (Glu or Asp) ligand. The replacement of the acidic ligand may be required to increase the nucleasephilicity of the bridging water molecule, so that hydrolysis of the resonance-stabilized C-N bond in NCβA can be achieved. There are to date only two other known members of the Acyl/M20 family with a corresponding zinc coordination, 1-N-carbamoylase from Sinorhizobium melliloti CECT 4114 (47) and allantooate amidohydrolase from Escherichia coli (44), which also hydrolyze non-peptidic bonds.

In the previously described SkβAS crystal structures, both zinc sites were fully occupied although no zinc was added to the enzyme except for the 1 mM ZnCl₂ present in purification buffers (16). This suggests stable binding of the metal ions at pH 6.0–6.5 of the crystallization condition, at which SkβAS retains only 20–30% of its maximum activity (Fig. 2a). In contrast, the electron density maps for the complex E159A_NCβA, crystallized at pH 8.75, indicated a significantly reduced occupancy (~0.3) of zinc site 2 (coordinated by Asp125, Glu160, and His421) in all active sites. The opposite behavior was observed for Sinorhizobium melliloti 1-N-carbamoylase; the crystal structure obtained at pH 4.6 did not contain any metal ions while that determined at pH 8.5 showed full occupancy of the metal sites (47).

Recent metal titration and crystallographic studies on E. coli methionine aminopeptidase, a member of the metallo-aminopeptidase family shown to possess a di-metal (Co²⁺ or Mn²⁺) center, revealed that the enzyme is fully active and able to bind ligands in the mono-metallated form, though a second metal ion can also be accommodated in the active site without causing enzyme inhibition (48). This raises the question whether the loss of the zinc ion in SkβAS is functionally significant or simply represents an artifact deriving from crystallization or purification procedures. Substrate binding to SkβAS is not hampered by the partial vacancy of zinc site 2 as seen in E159A_NCβA (described below). Metal content determinations indicated binding of 1–2 ions per subunit. An effect of purification procedure, pH of crystallization, enzyme conformational state or the mutation of a specific amino acid on metal center occupancy is unlikely because also E159A (pH 6.0) and a SkβAS-WT structure determined for crystals grown at pH 8.75⁰ have incomplete metal centers. Furthermore, all utilized enzyme batches were obtained with the same purification procedure. WT βA was subsequently obtained from crystals grown in presence of 50 μM ZnCl₂ (active site/zinc molar ratio of 1.6:1), and full occupancy is observed for both metal ions. Thus, the available data neither strongly support the significance of a mono-metallated enzyme state nor provide clear indications for how the zinc ion is lost. Further studies are required to determine whether the proposed catalytic mechanism (16) needs revision to allow functionality of mono-metallated SkβAS.

Substrate/Product Binding and Implications for the Catalytic Mechanism—The initial electron density map calculated for E159A_NCβA clearly indicated that the substrate is bound in the active site. It makes extensive contacts with the protein, forming a salt bridge and seven hydrogen bonds to residues R322A, H262A, N309, G396, and Q229, as well as a coordinating bond to zinc 2, which is present only in about one-third of the active sites of E159A_NCβA. These interactions are shown in Fig. 2b, and atom-to-atom distances are given in Table 2. Dimerization is essential for substrate binding because Asn309 and His262 participate in the formation of the active site of one monomer while belonging to the partner subunit. All involved

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4. Dobritzsch, unpublished, to be reported elsewhere.
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residues have previously been suggested to play a role in substrate binding (16), although not all interactions have been predicted correctly. For instance, His\textsuperscript{397} was thought to participate in coordination of the carboxyl group of NC\textbeta_A, but in reality it binds instead the carbamyl group. His\textsuperscript{397} was also suggested to play a role in substrate binding, and the crystal structure of the complex revealed that its side chain is located in close proximity to the ligand. However, the formation of hydrogen bonds to two water molecules and the side chain of Ser\textsuperscript{397} favors a ring orientation that excludes hydrogen-bonding with the substrate molecule (Fig. 2b). The superposition of WT\textunderscore{\textbeta} Ala onto the mutant complex places a carboxyl oxygen atom of Glu\textsuperscript{159} at a distance of 2.6–3.1 Å from the NC\textbeta_A-amide group (Fig. 2c), and the zinc-bridging water/hydroxyl ion can easily be reached after minor adjustments of the side chain conformation. Hence, the catalytic base is appropriately positioned for activation of the water by abstraction of a proton, which it then transfers to the substrates amide group, promoting cleavage of the carbon-nitrogen bond after nucleophilic attack of the bridging hydroxide at carbon 2.

The interactions of the reaction product \textbeta-alanine in WT\textunderscore{\textbeta} Ala are homologous to those of the corresponding atoms of the substrate in E159A\textunderscore{\textbeta} Ala (Fig. 2c). The only differences between the two structures are the presence of the Glu\textsuperscript{159} side chain and the completeness of the di-zinc center, including the bridging water, in the enzyme-product complex.
uct complex. The absence of the ligand in two of the four monomers per asymmetric unit and the poorer definition of electron density for the amino group of β-alanine indicates however a lower affinity of the enzyme for the reaction product. It also suggests that the crystallization conditions and not the binding of the reaction product stabilize the closed state of the enzyme in this complex.

During catalysis, attachment of the hydroxyl oxygen to atom C2 of the substrate causes a change in hybridization from sp² to sp³. It is likely that the newly attached group remains at the bridging position previously occupied by the hydroxyl (Fig. 2c). Positional changes of the carbamyl oxygen atom, which in the intermediate carries a negative charge, may be limited to a shift by less than 0.5 Å toward His262. This residue would therefore bind the intermediate stronger than the substrate, impairing that it has a function in transition state stabilization. In contrast, a rotation around the bond connecting atoms C2 and N1, e.g. to allow a bifurcate interaction of the gem-diol intermediate with the di-zinc center, would bring either atom O2 or N3 too close to the methyl group of Ala395.

Observed Functional and Structural Effects of the Mutations—SkβAS follows Michaelis-Menten kinetics at low substrate concentrations, but is inhibited by NCβA at concentrations >100 mM, which complicates the precise determination of the Michaelis constant significantly. The apparent $K_m$ is 50–70 mM (partly dependent on the enzyme batch), indicating low affinity of the native enzyme for the substrate NCβA. Most of the mutant enzymes exhibit very low residual activity and further decreased affinity for the substrate. Under these circumstances the $K_m$ values cannot be determined with sufficient accuracy, and therefore mainly the relative activities are discussed (Table 3).

Replacement of Ghu^{159} by alanine or aspartate resulted in a drastic loss of SkβAS activity. The crystal structure of SkβAS-E159A reveals an unaltered overall structure and undisturbed active site architecture, despite the deletion of the glutamate side chain. Open and closed states can be obtained for this mutant, while E159D crystallizes only under the conditions above described small changes in backbone conformation and a wider opening of the interdomain cleft. Although the crystals were grown in presence of 75 mM NCβA no substrate-associated density is observed near the di-zinc center. Instead, elongated electron density appears in three of the protomers per asymmetric unit at the position previously occupied by the arginine side chain. An NCβA molecule fits well into the density, interacting with Asp^{220}, Asn^{309}, Gly^{394}, Gly^{396}, and Trp^{251}, and this artificial binding site created by the mutation (supplementary Fig. S2). In the complexes SkβAS-E159A and Wt βAla, the distances between atoms Nγ1 and Nγ2 of Arg^{322} and the active site are reduced by ~5.0 and ~3.2 Å due to subunit closure. As a consequence, the carbamyl group of the substrate is moved into the vicinity of the di-zinc center, allowing catalysis to proceed (Fig. 2c).

The exchange of the zinc-ligating His^{226} by glutamate is also producing an inactive SkβAS variant, which in contrast to the other mutants could not be crystallized in either conformational state. The $k_{cat}$ of the mutant H262A is decreased 10–15 fold compared with the wild-type enzyme. Although the $K_m$ value determinations are not very precise due to the above mentioned reasons, the exchange appears to have little effect on substrate affinity (Table 3). In contrast, the replacement of His^{397} by asparagine has a more pronounced effect. The mutant enzyme retains only about 7% of the wild-type catalytic activity, and the $K_m$ is about 2–3 times increased. Crystals of SkβAS-H262A and H397N can be obtained under both crystallization conditions, but no structural data are currently available.

Mechanistic Implications—The almost complete loss of enzyme activity of SkβAS-E159A and -E159D is in agreement with the suggested crucial role of Ghu^{159} as a general base as well as with experimental data obtained for corresponding mutant enzymes of other Acyl/M20 family members (23, 47). The inactivity of SkβAS-E159D, in particular, may be explained by the inability of the aspartate to reach both the bridging water molecule and the amide group of the substrate, impairing proton abstraction as well as proton transfer. Both the open and closed conformational states of SkβAS-E159A could be crystallized, suggesting that the glutamate is not important for enzyme dynamics. The failure to produce crystals of the closed state and the high domain flexibility observed for the open conformation of SkβAS-E159D may therefore be caused by effects of the altered localization of the functional group on active site architecture.

The inactivity of SkβAS-R322A is consistent with results obtained for corresponding mutations of homologous proteins (45, 47) and indicates that the substrate is either not bound at all or in a mode incompatible with efficient hydrolysis. Thus Arg^{322} appears to be one of the key factors for substrate recognition. The ability of the mutant to form diffraction quality

### TABLE 2

| NCβA atom | Ligand atom | Distance (Å) |
|-----------|-------------|--------------|
| N3        | Zn1         | 2.76         |
|           | C229 - Oe1  | 3.09         |
|           | G396 - O    | 3.09         |
| O2        | Zn2         | 2.12         |
|           | H262 - Ne2  | 3.07         |
|           | C229 - Ne2  | 3.36         |
| N1        | G396 - O    | 2.70         |
| O41       | R322 - Nγ2  | 2.85         |
|           | N309 - Nγ2  | 2.72         |
| O42       | R322 - Nγ1  | 2.71         |
|           | G396 - N    | 2.59         |

### TABLE 3

| Enzyme       | $k_{cat}$ | Relative activity | $K_m$ | $k_{cat}/K_m$ |
|--------------|-----------|------------------|-------|---------------|
| Wild type    | 5.3 ± 0.62| 100              | ~60   | ~0.09         |
| SkβAS-E159A | 5.0 × 10⁻³| 0.09             | N.D.  | N.D.          |
| SkβAS-E159D | 5.0 × 10⁻³| 0.09             | N.D.  | N.D.          |
| SkβAS-R322A | 7.7 × 10⁻³| 0.14             | N.D.  | 0.02          |
| SkβAS-H262A | 0.47 ± 0.02| 8.9             | >50   | <0.009        |
| SkβAS-H397N | <5.0 × 10⁻³| 0.02             | N.D.  | N.D.          |

* Not determined.
However, considering that the active site of SkβAS is fine-tuned to harbor a histidine at position 226 it is more likely that the mutation of His397 to asparagine has a more dramatic effect on catalytic efficiency than the replacement of His262. The current lack of structural data in particular for the closed state makes it difficult to analyze whether changes in protein structure lead to activity loss. We can therefore only assume that the active site interactions involving the His397 side chain are important for nucleophilic attack on the NCβA-carbamyl carbon atom. However, considering that the active site of SkβAS is fine-tuned to harbor a histidine at position 226 it is more likely that the substrate for such a role. It is thus quite surprising that the lack of structural data for the mutant SkβAS-H226A hampers a precise determination of the reasons for the dramatic loss in catalytic activity. It is possible that the mutant is unable to lower the pK_a of the bridging water molecule enough for nucleophilic attack on the NCβA-carbamyl carbon atom. However, the significantly smaller decrease in k_cat upon mutation. Additional interactions of the oxyanion e.g. with Gln229 and the di-zinc center may sufficiently stabilize the transitions state even in absence of the imidazole group. The modest activity loss also implies that active site architecture, protein dynamics, and stability are not strongly affected.

Previously, we implicated His397 instead of His262 in transition state stabilization. However, in E159A_NCβA its imidazole ring is too distant from the carbamyl oxygen of the substrate for such a role. It is thus quite surprising that the mutation of His397 to asparagine has a more dramatic effect on catalytic efficiency than the replacement of His262. The current lack of structural data in particular for the closed state makes it difficult to analyze whether changes in protein structure lead to activity loss. We can therefore only assume that the active site interactions involving the His397 side chain are important for correct placement and/or orientation of substrate binding residues, and possibly also for completeness or maintenance of the required electronic state of the di-zinc center.

Crystals of the native, open-state enzyme (16) were suggested His262 to be involved in anchoring of the NCβA-carboxyl group. However, recent site-directed mutagenesis studies performed on Acyl implicated the corresponding His206 in catalysis, as its replacement by asparagine or alanine caused an over 11000-fold or 560-fold reduction in k_cat/K_m, respectively (23, 45). Structure analysis of the complex E159A_NCβA supports an involvement of the histidine in catalysis rather than substrate binding. It most likely forms a hydrogen bond to the oxyanion of the tetrahedral intermediate, partly compensating its negative charge. However, His262 is not similarly essential for catalysis as the corresponding Acyl residue as indicated by the significantly smaller decrease in k_cat upon mutation. Additional interactions of the oxyanion e.g. with Gln229 and the di-zinc center may sufficiently stabilize the transitions state even in absence of the imidazole group. The modest activity loss also implies that active site architecture, protein dynamics, and stability are not strongly affected.

Crystals at the pH 8.75 condition implies that the closed state is reached and sufficiently stabilized even in absence of the Arg222 side chain.
Crystal Structures of β-Alanine Synthase Ligand Complexes

Based on the analysis of all structural and functional data accumulated for SkβAS, we hypothesize that the open and closed conformational states coexist in a dynamic equilibrium, with the open state allowing ligand binding and release and the closed state promoting catalysis. Therefore, no specific mechanism may be required to trigger the transformation from one state to the other. Although no direct evidence has yet been found, most observations fit such a hypothesis, e.g. the domain flexibility associated with the open state even after packing into a crystal lattice. It could also explain the low affinity of SkβAS for its substrate, because NCβA entry into the active site is only possible when the enzyme is in a conformational state that provides few opportunities for tight interactions.

Comparison of the Closed States of SkβAS and PepV—The superimposition of the dimer of E159A_NCβA with the crystal structure of PepV (PDB-ID 1fw) (24), the only other AcyI/M20 family member for which a closed ligand-bound state has been observed, results in an r.m.s.d. of 1.93 Å for 275 equivalent Cα atoms. This unspecific amino dipeptidase exists as a monomer but its so-called lid domain is extended by ~90 residues, which remarkably well mimic the structure, location and orientation of the dimerization domain from the partner subunit in the closed state of the dimeric SkβAS (Fig. 3a). PepV recognizes and fixes the dipeptide backbone, while the side chains of the substrate are not specifically probed. To a certain extent, NCβA resembles the chemical structure of a dipeptide backbone, and it is hence not entirely surprising that most of the zinc- and substrate-binding residues are conserved between both enzymes. The transition state mimic AspΨ[PO2CH2]AlaOH bound to PepV is only slightly shifted with respect to the location of NCβA in the active site of E159A_NCβA (Fig. 3b). It introduces the hydroxyl group representing the catalytically attached water molecule as the bridging moiety between the zinc ions. The arginine, asparagine, and histidine corresponding to Arg322, Asn309, and His397 are moved ~1 Å further into the active site, as an adjustment to the shorter C-terminal α-amino acid of the dipeptide compared with the β-amino acid moiety of NCβA. Residues surrounding the N-terminal amino acid of the PepV substrate are not conserved in SkβAS, reflecting the structural differences between the substrates. In fact, the cavity available for this part of the PepV substrate in SkβAS is completely filled by the side chains of His397, Gln229, His236, and Ala395. Furthermore, while the substrate binding cavity of PepV extends to the protein surface, that of SkβAS is completely disconnected from the surrounding solvent when the closed conformational state is reached. The bulky side chain of Trp325 (a threonine in PepV) and the extension of the loop following Glu160 as compared with the corresponding loop in the peptidase are mostly responsible for the blockage of the entrance. The limited size of the active site cavity in SkβAS implies that even small dipeptides could not be bound properly to allow hydrolysis of a peptide bond. Besides NCβA and N-carbamyl-β-amino isobutyrate, no potential other N-carbamylated substrates of SkβAS could thus far be identified, which is in agreement with the observed restricted space that would be available for their binding.

CONCLUSIONS

The structures of the enzyme complexes presented here provide the first picture of the closed state-conformation of SkβAS which is expected to apply also to other dimeric enzymes of the Acyl/M20 family. We could thus provide experimental evidence that domain closure movements are required for catalysis. It is achieved by a ~30° rigid body rotation of the catalytic domain with respect to the dimerization domain, and does not involve significant conformational changes within the distinct domains. It is therefore likely that catalysis can occur independently in the two active sites per homodimer. Substrate-binding and putative catalytic residues could be pinpointed by analysis of the complexes E159A_NCβA and WT_βAla. The suggested roles for some of these residues were confirmed by site-directed mutagenesis experiments, those of His262 and His397 could be corrected. The crystal structures of the described SkβAS complexes may provide the means for accurate modeling of the closed states of other Acyl/M20 family proteins. As some of them are of therapeutic significance or promising targets for antimicrobial agents, such models could be used as frameworks for structure-assisted drug design.

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