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Identification of the catalytic residues in the double-zinc aminopeptidase from *Streptomyces griseus*

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Abstract The aminopeptidase from *Streptomyces griseus* (SGAP) has been cloned and expressed in *Escherichia coli*. By growing the cells in the presence of 1 M sorbitol at 18 °C, the protein was obtained in a soluble and active form. The amino acid sequence of the recombinant SGAP contained four amino acids differing from the previously published sequence. Re-sequencing of the native protein indicated that asparagines 70 and 184 are in fact aspartic acids as in the recombinant protein. Based on the crystal structure of SGAP, Glu131 and Tyr246 were proposed to be the catalytic residues. Replacements of Glu131 resulted in loss of activity of 4–5 orders of magnitude, consistent with Glu131 acting as the general base residue. Mutations in Tyr246 resulted in about 100-fold reduction of activity, suggesting that this residue is involved in the stabilization of the transition state intermediate.

Keywords: Aminopeptidase; Soluble expression; Sorbitol; Catalytic mechanism; *Streptomyces griseus*

1. Introduction

Aminopeptidases (APs) are a group of exopeptidases that catalyze the cleavage of amino acids from the amino-terminus of proteins and peptides. APs activity is associated with many biological functions such as protein maturation, protein degradation, hormone level regulation and cell-cycle control. Therefore, these enzymes play an important role in many pathological conditions including cancer, cataract, cystic fibrosis, leukemia and HIV infection [1–6].

APs can be classified into families and clans based on their sequence homology. Many known clan M APs are metallopeptidases [7,8] and family M28 APs includes bacterial and human enzymes that accommodate two Zn ions in their active site. Some of the bacterial APs in this family are *Streptomyces griseus* aminopetidase (SGAP), *Aeromonas proteolytica* aminopeptidase (AAP) and the putative aminopeptidase from *Bacillus subtilis* (BSAP). Family M28 includes also human proteins such as the prostate specific membrane antigen (PSMA) and the glutamate carboxypeptidase II (GCP-II) [9,10]. The catalytic domains of these two medically important human proteins share ~30% identity with bacterial APs, including the conserved catalytic residues and the Zn binding ligands [11–13].

To date, very few double-Zn APs have been characterized and limited mechanistic data are available on this class of metalloaminopeptidases. Revealing the mechanism of co-catalytic active site APs is particularly important in light of the potential for designing new inhibitors acting as pharmaceuticals [14,15]. Bacterial APs are also excellent candidates for structure–function analysis, since they can be easily produced and purified as recombinant proteins.

SGAP is a 30 kDa, thermostable, double-zinc AP that prefers large hydrophobic amino-terminus residues in its substrates [16,17]. We have recently solved the crystal structure of SGAP together with its reaction products [11–13]. Based on the crystallographic results and molecular dynamics simulations, a catalytic mechanism involving Glu131 and Tyr246 was proposed [12] (Fig. 1). Glu131 is suggested to act as a general base activating a water molecule for a nucleophilic attack on the substrate carbonyl carbon, and as a proton "shuttle" during the catalytic cycle, as was first proposed for thermolysin [18]. Tyr246 possibly polarizes the carbonyl carbon of the N-terminal substrate [12] (Fig. 1).

In this study, we describe the cloning and site directed mutagenesis of the *sgap* gene from *S. griseus*. The resulting catalytic mutants were purified and their catalytic properties were determined, providing for the first time biochemical confirmation to their catalytic role.

2. Materials and methods

2.1. Cloning of the sgap gene

Based on the published amino acid sequence of SGAP [19], several degenerative primers were designed for amplification of the gene via PCR using *S. griseus* (DSM 40855) chromosomal DNA as a template. The best results were obtained with a C-terminal primer that included the last 22 amino acids of the gene. Both primers were designed in a way that enabled the cloning of the gene into the T7 polymerase expression vector pET9d (Novagen). The N-terminal primer (5′-

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Abbreviations: Leu-*p*NA, Leucine-*para*-nitroanilide; SGAP, *Streptomyces griseus* aminopeptidase

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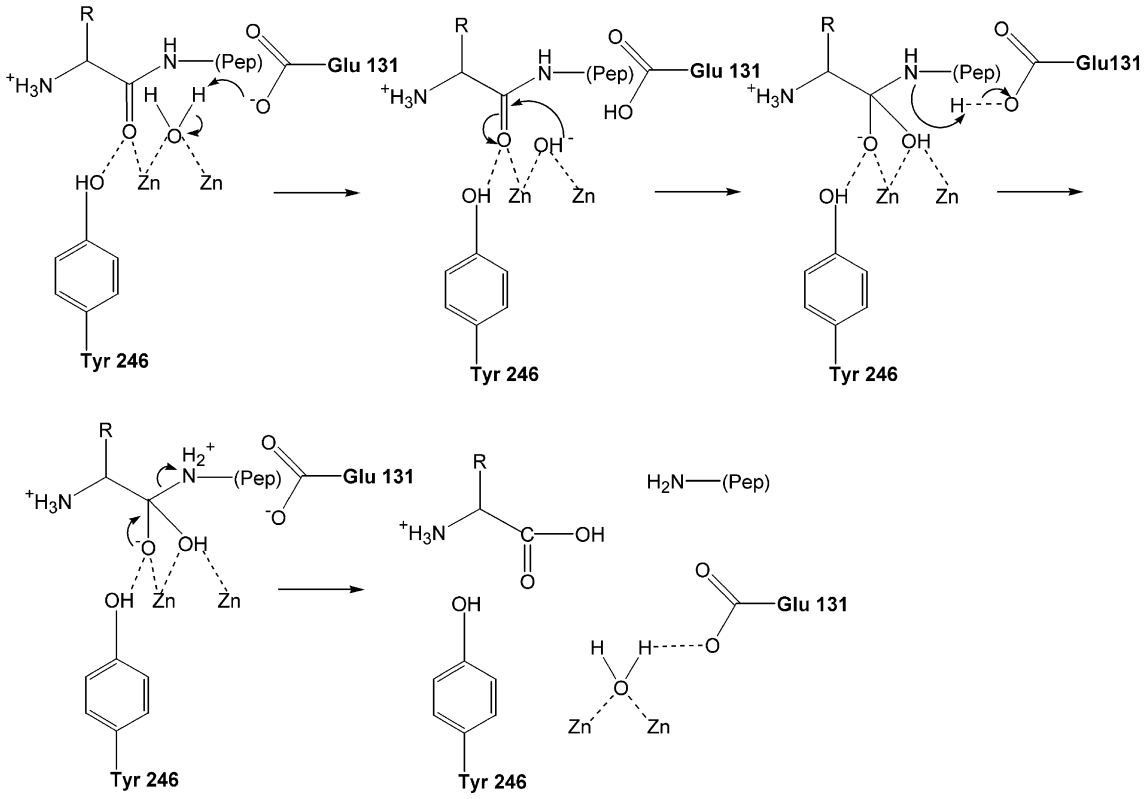

Fig. 1. The proposed catalytic pathway of SGAP. An acidic residue (Glu131) activates a zinc-bound water molecule and an additional residue (Tyr246) polarizes the carbonyl carbon and stabilizes the transition state. Dashed lines indicate stabilizing interactions and/or hydrogen bonds in the catalytic pathway; (Pep) – the incoming peptide/protein substrate.

GGAATTCCATGGCGCCGGACATCCCGCTGGC-3′) included the ATG translated start codon inside the *Nco*I (CCATGG) restriction site. The C-terminal primer (5′-GCGGATCCTTAGGTTGGTGGGTTCGCCGGTGCCGCTGCTCAGGGTCCAAATGGCATGTGCTGCTGCATCGCTGTTGCGGTCGAGGGCGGTGTCGTTGATG-3′) included *Bam*HI site (GGATCC) at the end of the gene. Following PCR, the amplified product was digested with *Nco*I and *Bam*HI, cloned into pET9d, and the SGAP gene was sequenced. The protein sequences of the recombinant and the native enzyme (purified from pronase) were also verified using electrospray ionization ion trap mass spectrometry (The Smoler Proteomic Center, Technion).

2.2. Expression and purification of SGAP

E. coli BL21(DE3) (pET9d-SGAP) cells were grown in Luria Broth (LB) containing kanamycin (25 μg/ml) for a few hours, at 37 °C, and then transferred to Terrific Broth (TB) [20] supplemented with sorbitol (1 M), betain (2.5 mM), phosphate buffer, pH 7 (50 mM), and kanamycin (25 μg/ml), (500 ml in 2-l shake flasks, shaken at 180 rpm, 37 °C). At a culture density of 0.6 OD_{600}, the growth temperature was reduced to 18 °C and isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 100 μM.

Following 40 h of growth (final OD_{600} of 12–15), cells from 1-l culture were harvested (7000 rpm for 10 min), washed twice with 30 mM Tris–HCl, pH 8, and 1 mM $CaCl_2$, and then resuspended in 45 ml of 25 mM Tris–HCl, 3 mM NaCl, 1 mM $CaCl_2$, and 0.05 mM $ZnCl_2$, pH 9. The cells were disrupted by two passages through a French® press (Spectronic instruments, Inc., Rochester, NY, USA) at room temperature. The cell extract was centrifuged (13 000 rpm for 20 min at 4 °C), and the soluble fraction was heat-treated (50 °C, 20 min) and centrifuged again. The soluble fraction containing the recombinant SGAP was diluted 10-fold with water to decrease the ionic strength and the pH was adjusted to 9.0. The sample was then loaded on a HiPrep™ Q Sepharose (functional group $-N^+(CH_3)_3$) Fast Flow column XK 50/20 running at 40 ml/min with 3 mM NaCl, 25 mM Tris–HCl, 1 mM $CaCl_2$, and 0.05 mM $ZnCl_2$, pH 9, and eluted with 7 mM NaCl in the same buffer (AKTA explorer (Pharmacia)). The enzyme appeared as a distinct protein peak, which was collected and used for biochemical characterization and crystallization experiments. Protein concentrations were determined by the Bradford method, with bovine serum albumin as a standard [21].

2.3. Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuickChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The mutagenic primers for all the mutants were as follows (Mutated nucleotides are shown in bold):

E131A: 5′-CCTGGTGGGGCGC**C**G**C**GGAGCTGGG-3′ and 5′-CCCAGCTCC**GC**G**G**CGCCCCACCAGG-3′; E131Q: 5′-CTGGTGGGGCGCGG**C**AGGAGCTGGGCC-3′ and 5′-GGCCCAGCTCCT-**G**CGCGCCCCACCAG-3′; E131D: 5′-GGTGGGGCGCGGA**T**GAGCT**C**GGCCTGATC-3′ and 5′-GATCAGGCC**G**AGCTC**A**TCCGCGCCCCACC-3′; Y246A: 5′-GCCTTCGACCG**G**TGC**G**CCCACTCCTCGTGC-3′ and 5′-GCACGAGGAGTGG**GC**GCA**C**CGGTCGAAGGC-3′; Y246F: 5′-GCCTTCGACCG**G**TGCT**T**CCACTCCTCGTGCG-3′ and 5′-CGCACGAGGAGTGGA**A**GCA**C**CGGTCGAAGGC-3′; Y246S: 5′-GCCTTCGACCG**G**TGCT**C**CCACTCCTCGTGCG-3′ and 5′-CGCACGAGGAGTGG**G**AGCA**C**CGGTCGAAGGC-3′. Y140F: 5′-GGCTCGAAGT**T**CTACGT**T**AACAACCTGCCGTCCG-3′ and 5′-CGGACGGCAGGTTGTT**A**ACGTAGA**A**CTTCGAGCC-3′.

All of the mutated genes were sequenced to confirm that only the desired mutations were inserted. The mutated proteins were overexpressed and purified as the wild type protein.

2.4. Kinetic studies

Kinetic studies were carried out by a continuous assay using L-leucine-*para*-nitroanilide (leu-*p*NA) (Sigma) as a substrate. The continuous reactions were performed at 30 °C in a 1-ml cuvette. The assay solution contained 650 μl of 50 mM MOPS, pH 8, 1 mM $CaCl_2$, 0.05 mM $ZnCl_2$ and 1 mg/ml BSA together with an appropriate diluted enzyme and substrate concentrations ranging from 0.1 to 10 K_m. The increase in absorbance at 405 nm was followed continuously using an

Ultrospec 2100 spectrophotometer (Pharmacia). The extinction coefficient at 405 nm used for *para*-nitroanilide was $\Delta\epsilon = 9.65\ mM^{-1}\ cm^{-1}$ at pH 8. The catalytic constants, K_m and k_{cat}, were determined by nonlinear regression analysis with GraFit 5.0 [21].

3. Results and discussion

3.1. Cloning and sequence analysis of SGAP

The SGAP gene from *S. griseus* was cloned into pET9d via PCR using degenerative primers [19] and was overexpressed in *E. coli* cells. The sequence of the gene indicated that the recombinant SGAP is identical to the native protein purified from pronase [19], except for four amino acids. The recombinant SGAP contained Tyr140, Arg240, Asp70 and Asp184, instead of Phe140, Gln240, Asn70 and Asn184 that appear in the native protein. To verify again the amino acid sequence of the native protein (purified from pronase), it was subjected to electrospray ionization ion-trap mass spectrometry (EIMS). The EIMS results indicated that the native protein contains in fact aspartic acids in positions 70 and 184, and not Asn as was published before. Thus, the recombinant SGAP (rSGAP) obtained from *S. griseus* (DSM 40855) differs only in two amino acids (Phe140 and Gln240) from the native SGAP obtained from the common commercial source (pronase).

3.2. Optimizing the expression of SGAP

Initial attempts to express the SGAP protein in *E. coli* resulted in very low yields of soluble protein. To improve expression and solubility of the SGAP gene product, several methods were tested including the use of various *E. coli* strains and expression vectors, and changing the growth temperature to 18 °C. None of these procedures resulted in any significant improvement of expression. Finally, we tested the effect of sorbitol in the growth media [22]. Indeed, growing the cells under high osmotic pressure resulted in over 500-fold increase in cell-associated SGAP activity. Sorbitol is a compatible solute, which raises the osmotic pressure and presumably induces production of intracellular osmoprotectants (such as trehalose) or other stress-response mechanisms that may affect the folding kinetics of the recombinant protein. Several concentrations of sorbitol (0.2–1.5 M) in LB and TB media were tested at several growth temperatures (17–37 C). The best results were obtained by growing the cells on TB with 1 M sorbitol at 18 °C for 40 h under IPTG induction.

3.3. Purification of SGAP

SGAP was purified from the crude extract by a combination of heat treatment and anion exchange chromatography. Although *S. griseus* is not a thermophilic bacterium, the enzyme is heat stable [17]. The sample was first heat-treated for 20 min in 50 °C resulting in recovery of 74% and then was subjected to anion exchange chromatography on HiPrep™ Q Sepharose Fast Flow (Fig. 2). Most of the enzyme activity was eluted as a single peak at 7 mM NaCl. This purification procedure resulted in a total recovery of 74% and a 28-fold purification factor, giving a specific activity of 830 U/mg protein (Table 1). This specific activity is comparable to what was obtained from the pronase-purified SGAP (about 500 U/mg protein) [17]. The overall yield was ~50 mg of pure SGAP from 1-l culture. A recent study by Ni et al. [23] reported the large scale purification procedure of a recombinant SGAP, which resulted in a very low yield of 2.57 mg purified SGAP from 16-l culture [23]. Thus, the yield obtained in this study is over 300-fold higher.

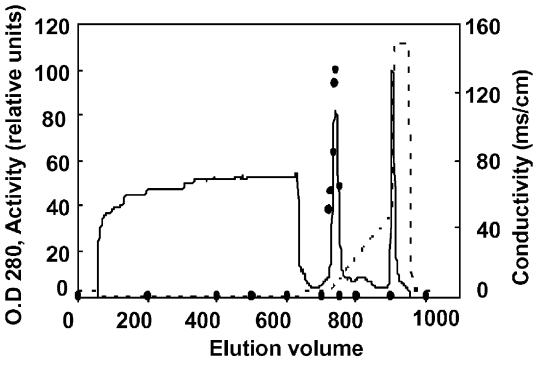

Fig. 2. Purification of SGAP. Chromatogram of HiPrep™ Q Sepharose Fast Flow anion exchange. OD 280 nm (solid line) and activity (closed circles) (both in relative units), conductivity (dashed line) in ms cm^{-1}.

3.4. Testing catalytic mutants

One of the most direct approaches to evaluate the role of proposed catalytic residues is to replace them by site directed mutagenesis and test the properties of the resulting catalytic mutants [24]. Due to the low activity of the catalytic mutants, relatively large amounts of the purified protein are required. The successful production of the recombinant SGAP in high yields allowed us to test various catalytic mutants. Based on the high resolution structure of SGAP, a general catalytic pathway was proposed (Fig. 1) [12]. These studies suggest that the conserved Glu131 and Y246 residues are directly involved in the catalytic mechanism of the enzyme. Glu131 probably acts as a general base by promoting the nucleophilic attack of the zinc-bound water on the substrate carbonyl carbon and as a proton shuttle during the catalytic cycle. This residue may also be involved in binding the substrate and stabilizing the tetrahedral transition state. SGAP and AAP share an amino acid sequence homology of only 30% [19]. Based on their crystal structures, it appears that they contain a comparable catalytic core [13] and share a similar double-zinc coordination [11]. By comparing the SGAP complex structures with reaction products to the complex of AAP with *p*-iodo-D-phenylalanine hydroxamate, it could be seen that they both have a significant

Table 1
Purification of recombinant SGAP

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor (fold)
Crude extract[a]	250	1.5×10^3	1.9×10^5	12	100	1
Heat treatment	185	5×10^3	1.5×10^5	30	74	2.5
Anion exchange	285	180	1.5×10^5	830	74	28

[a] The crude extract was obtained from 4-l culture.

interaction of glutamate (Glu131 in SGAP and Glu151 in AAP) with one of the zinc-bound oxygen atoms of the analog [12,25]. Glu151 was proposed to assist in deprotonation of the metal bound water as to create the nucleophile hydroxide of the initial catalytic reaction of AAP [14].

To test the role of Glu131 in SGAP, it was replaced by site directed mutagenesis to either Gln, Asp or Ala. Gln is a non-ionizable amino acid with a terminal amide group, allowing it to form hydrogen bonds, but it cannot act as a general base. Asp is shorter by one carbon from Glu, thus this replacement should affect the efficiency of deprotonating the zinc-bound water molecule. The Glu131Ala replacement resulted in over five orders of magnitude decrease in k_{cat} as compared to the wild type, but did not significantly affect K_m (Table 2). This very large reduction in activity is consistent with Glu131 being the general base residue. The two other replacements, Glu131Gln and Glu131Asp, also resulted in a large reduction in k_{cat} of about four orders of magnitude, reflecting the fact that these residues may function to some extent as proton donors. Similar level of reduction in activity was obtained with other general base catalytic mutants of metallopeptidases, including the monozinc APA [26–28], peptide deformylase [29], neutral endopeptidase (EC 3.4.24.11) [30], human matrilysin [31], leukotriene A_4 hydrolase [32], glutamate carboxypeptidase II [33] and thermolysin [24,34].

The proposed role of Y246 in SGAP is to polarize the carbonyl group of the N-terminal substrate and to make it more accessible to a nucleophilic attack by the activated water molecule. In addition, this residue may also stabilize the tetrahedral intermediate. Tyr246 was replaced to either Ser, Ala or Phe, and all the replacements resulted in about two orders of magnitude decrease in activity (Table 2). The 100-fold decrease of the k_{cat} value suggests that the phenolic hydroxyl of Tyr246 plays an important role in substrate hydrolysis. Studies with monozinc metalloproteinases, in which a similar functional residue was replaced, also resulted in a 100-fold reduction in k_{cat} [27,31,35]. The reduction in activity of the Tyr246 replacements is again consistent with the role of this residue in polarizing the carbonyl group of the peptide bond and stabilizing the transition state by hydrogen bond interaction with one of the tetrahedral intermediate oxygen atoms.

Aligning SGAP to other family M28 peptidases reveals that the Tyr246 is almost fully conserved and located near a conserved His residue that is suggested to act as a Zn ligand [12]. PSMA appeared to contain an active site resembling that of SGAP. Replacement of the equivalent Tyr552 to Phe in PSMA reduced V_{max} 10-fold [33]. Tyr was also proved to play a critical role in the catalytic reaction and in substrate binding of bovine carboxypeptidase A. Replacing this residue to Ala or Phe resulted in 10-fold decrease of k_{cat} and 4–11-fold increase in K_m [36]. These findings indicate again that this residue participates in catalysis. Other APs contain alternative amino acid residues in their active site, such as His, that are involved in transition state binding [35]. Mutation in the His residue resulted in 100-fold reduction of k_{cat} similar to what was obtained with the SGAP Tyr246 mutants in this study.

In conclusion, the successful production of active soluble recombinant SGAP allowed us for the first time to test the role of the structure based catalytic residues of double-zinc AP. The kinetic results provide further evidence that Glu131 acts as a general base and that Tyr246 is probably involved in stabilization of the tetrahedral intermediate. The recombinant proteins could also be crystallized. Preliminary results suggest that the SGAP E131D mutant crystallizes in the monoclinic space group $P2_1$.

Table 2
Kinetic parameters for hydrolysis of leu-*p*NA by SGAP and its catalytic mutants

Enzyme	k_{cat} (s^{-1})[a]	K_m (mM)[a]	k_{cat}/K_m ($s^{-1}\,mM^{-1}$)
WT	3.9×10^2	0.57	689
E131A	3.3×10^{-3}	0.33	0.01
E131D	4×10^{-2}	0.4	0.1
E131Q	6×10^{-2}	0.39	0.16
Y246A	2.2	0.78	2.8
Y246F	5.6	0.33	17.1
Y246S	8	0.7	11.6

[a] The reaction was performed at 30 °C in a continuous assay. Reaction solution contained 50 mM MOPS, pH 8, 3 μM $ZnCl_2$ and the appropriate enzyme and substrate concentrations.

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