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Biochemical and mutational characterization of N-succinyl-amino acid racemase from Bacillus stearothermophillus CECT49

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Corresponding Author:	Sergio Martínez-Rodríguez, Ph.D. SPAIN
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	
Corresponding Author's Secondary Institution:	
First Author:	Pablo Soriano-Maldonado
First Author Secondary Information:	
Order of Authors:	Pablo Soriano-Maldonado
	Montserrat Andújar-Sánchez
	Josefa María Clemente-Jiménez
	Felipe Rodríguez-Vico
	Francisco Javier Las Heras-Vázquez
	Sergio Martínez-Rodríguez, Ph.D.
Order of Authors Secondary Information:	
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Title Page with all authors' contact info

Pablo Soriano-Maldonado[a,b], Montserrat Andújar-Sánchez[a,b], Josefa María Clemente-Jiménez[a,b], Felipe Rodríguez-Vico[a,b], Francisco Javier Las Heras-Vázquez[a,b], Sergio Martínez-Rodríguez[a,b,*,#].

[a] *Dpto. Química y Física, Universidad de Almería, Campus de Excelencia Internacional Agroalimentario, ceiA3, 04120, Almería, Spain.*

[b] *Centro de Investigación en Biotecnología Agroalimentaria, BITAL. Almería, Spain.*

[*] **Corresponding author:** Sergio Martínez-Rodríguez

Dpto. Química y Física.

Universidad de Almería.

Edificio CITE I, Carretera de Sacramento s/n.

04120 La Cañada de San Urbano, Almería (Spain)

srodrig@ual.es

[#]Actual address, Department of Physical Chemistry, University of Granada, 18071, Granada, Spain. sergio@ugr.es

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Biochemical and mutational characterization of *N*-succinyl-amino acid racemase from *Geobacillus stearothermophilus* CECT49

Keywords: *N*-succinyl-amino acid racemase, *N*-acyl-amino acid racemase, *N*-acetyl-amino acid racemase, acylase process, amino acid.

Abstract

N-succinyl-amino acid racemase (NSAAR), long referred to as *N*-acyl- or *N*-acetyl-amino acid racemase, is an enolase superfamily member whose biotechnological potential was discovered decades ago, due to its use in the industrial dynamic kinetic resolution methodology first known as "Acylase Process". In previous works, an extended and enhanced substrate spectrum of the NSAAR from *Geobacillus kaustophilus* CECT4264 toward different *N*-substituted amino acids was reported. In this work, we describe the cloning, purification and characterization of the NSAAR from *Geobacillus stearothermophilus* CECT49 (GstNSAAR). The enzyme has been extensively characterized, showing a higher preference toward *N*-formyl-amino acids than to *N*-acetyl-amino acids, thus confirming that the use of the former substrates is more appropriate for a biotechnological application of the enzyme. The enzyme showed an apparent thermal denaturation midpoint of 77.0±0.1 ºC and an apparent molecular mass of 184±5 kDa, suggesting a tetrameric species. Optimal parameters for the enzyme activity were pH 8.0 and 55-65 ºC, with Co^{2+} as the most effective cofactor. Mutagenesis and binding experiments confirmed K166, D191, E216, D241 and K265 as key residues in the activity of GstNSAAR, but not indispensable for substrate binding.

Introduction

N-succinyl-amino acid racemase (NSAAR), long referred to as *N*-acyl- or *N*-acetyl-amino acid racemase (NAAR)[1], is an enolase superfamily member whose biotechnological potential was discovered decades ago, due to its use in the industrial dynamic kinetic resolution (DKR) methodology first known as "Acylase Process" [1]. Though this enzyme has been mainly studied because of its *N*-acetyl-amino acid racemase activity [1-9], its catalytic promiscuity attracted the attention of several scientists, whose work on the evolution and real classification of these enzymes [10-16] led them to propose its classification as a NSAAR [16,17]. This enzyme belongs to the mechanistically diverse enolase superfamily, and in fact, it is an example of real catalytic promiscuity, since it is active toward many different substrates [10].

Despite a high number of patents describing the use of NSAAR/NAAR [18-24], and works related to the isolation of microorganisms showing this activity [2, 25], only a small number of NSAAR/NAAR enzymes have been described in detail in the literature. In fact, only the enzymes belonging to *Amycolatopsis* [1,2,3,6,7,26], *Deinococcus* [8,27-30], *Streptomyces* [3] and *Geobacillus* [9,16,31-33] have been characterized to different extents. The crystallographic structure of *Thermus thermophilus* HB8 NAAAR has also been reported, but its activity has not been described [34]. Besides *N*-acetyl-amino acids, NSAAR/NAAR enzymes have also been reported to racemize other *N*-substituted amino acids to very different extents [3,4,6,7,9,16,32,33,35]. The recombinant NSAAR from *Geobacillus kaustophilus* CECT4264 (GkaNSAAR) proved to racemize *N*-formyl-amino acids more efficiently than the industrially used acyl- derivatives [31], allowing us to develop a more general

[1]We will use the three names indistinctly during this work, to try to maintain the original nomenclature used in the corresponding papers.

and efficient DKR method based on the "Acylase Process" (namely Amidohydrolase Process) by coupling it with a stereospecific L-*N*-carbamoylase (Figure 1) [32,33]. Motivated by the potential of NSAAR enzymes in DKR processes for optically pure D- and L-amino acid production [28-30,36], a recent paper highlights the efforts carried out to engineer improved NSAAR enzymes for this purpose [35]. Accordingly, it seems clear that finding new enzymes with improved characteristics or broader substrate spectrum would enhance this DKR methodology, since NSAAR enzymes constitute the limiting step for reaction in the bienzymatic tandems where this enzyme has been applied [28-30,32,33,35].

In this work, we have cloned, overexpressed, purified and characterized the NSAAR belonging to *Geobacillus stearothermophilus* CECT49 (GstNSAAR), specifically evaluating its *N*-formyl-amino acid racemase activity, since this activity has not been previously studied in detail for any NSAAR enzyme. In order to provide additional information on the mechanism and enzymatic features of NSAAR enzymes, we specifically altered five amino acids in the catalytic center of the enzyme to investigate their biochemical role in binding and catalysis.

Material and Methods

Materials

All chemicals were of analytical grade and were used without further purification. Restriction enzymes and T4 DNA ligase were purchased from Roche Diagnostic S.L. (Barcelona, Spain). Kapa-Hifi polymerase was from Cultek S.L.U. (Madrid, Spain). Primers were from IDT (Biomol, Spain). TALON^{TM} metal affinity resin was purchased from Clontech Laboratories, Inc. Racemic and optically pure amino acids and *N*-acetyl-

methionine were from Sigma Aldrich Quimica S.A. (Madrid, Spain). The *N*-carbamoyl-, *N*-succinyl- and *N*-formyl-amino acids were synthesized according to previous works [9, 32-33].

Microbes and culture conditions

Geobacillus stearothermophilus CECT49 *and Deinococcus radiodurans* CECT833 were used as possible donors of different *N*-succinyl-amino acid racemase (*nsaar*) genes. *Geobacillus stearothermophilus* CECT49 was grown on nutrient broth/agar I plates (1% peptone, 0.5% beef extract, 0.5% NaCl, pH 7.2, 1.5% Agar) at 55 ºC (24 h). *Deinococcus radiodurans* CECT833 was grown on *Corynebacterium* broth/agar plates (1% casein peptone (tryptic digest); 0.5% yeast extract, 0.5% glucose, 0.5% NaCl, 1.5% Agar) at 30 ºC (72 h). *Escherichia coli* DH5α was used to clone the different putative *nsaar* genes, and *E. coli* BL21 (DE3) to overexpress the proteins.

Cloning of N-succinyl-amino acid racemase (nsaar) genes

A single-colony isolate of *Geobacillus stearothermophilus* CECT49 *and Deinococcus radiodurans* CECT833 strains was chosen for DNA extraction by a boiling procedure. A sample of the supernatant containing genomic DNA (5 µl) was used to amplify the different fragments encoding for putative *nsaar* genes by PCR. Each of the obtained PCR fragments was purified from agarose gels using QIAquick (Qiagen) and then subcloned using a StrataClone™ PCR Cloning Kit (Stratagene). The isolated sub-cloning plasmids were purified using QIAprep Spin miniprep kit (Qiagen), and digested using *Nde*I/*Xho*I (*Deinococcus radiodurans* CECT833 gene, *drcnsaar*) or *Sac*I/*BamH*I (*Geobacillus stearothermophilus* CECT49 gene, *gstnsaar*). The digested fragments were purified from agarose gel using QIAquick (Qiagen), and then the *drcnsaar* gene

was ligated into *Nde*I/*Xho*I site of pET22b+ plasmid (Novagen), and *gstnsaar* gene into the *Sac*I/*Bam*HI site of the rhamnose-inducible expression vector pJOE4036.1 [37]. The resulting constructions allowed the production of the *Geobacillus stearothermophilus* CECT49 NSAAR (GstNSAAR) and *Deinococcus radiodurans* CECT833 (DrcNSAAR) enzymes with a C-terminal His_6-tag. The cloned DNA fragments were sequenced using the dye dideoxy nucleotide sequencing method in an ABI 377 DNA Sequencer (Applied Biosystems). Translated sequences were aligned and compared with other available amino acid sequences using the Basic Local Alignment Search Tool (BLAST, NCBI). Clustal W-XXL was used for alignment [38].

Expression of GstNSAAR and DrcNSAAR

The different plasmids were transformed into *E. coli* BL21 (DE3) and then grown in LB medium supplemented with 100 $\mu g \cdot ml^{-1}$ of ampicillin. A single colony was transferred into 10 ml of LB medium supplemented with 100 $\mu g \cdot ml^{-1}$ of ampicillin in a 100 ml flask, and these cultures were incubated overnight at 37 ºC with shaking. 500 ml of LB supplemented with 100 $\mu g \cdot ml^{-1}$ of ampicillin were inoculated with 5 ml of the overnight culture in a 2 l flask. After 2 h of incubation at 37 ºC with vigorous shaking, the OD_{600} of the resulting cultures was 0.3-0.5. For expression induction of the putative *gstnsaar* and *drcnsaar* genes, L-rhamnose (0.2%) or isopropyl-β-thio-D-galactopyranoside (IPTG, 0.2 mM) were added to the cultures, respectively. The cultures were continued for a further 6 hours at 32 ºC and 34 ºC, respectively. The cells were collected by centrifugation (Beckman JA2-21, 7,000 g, 4 ºC, 20 min), and stored at -20 ºC until use. Overexpression of GkaNSAAR enzyme was carried out as previously described [32].

Purification of GstNSAAR, DrcNSAAR and GkaNSAAR enzymes

Recombinant *E. coli* cells were resuspended in 30 ml wash buffer (300 mM NaCl, 0.02% NaN_3, 50 mM sodium phosphate pH 7.0). The cell walls were disrupted in ice by sonication using a UP 200 S Ultrasonic Processor (Dr. Hielscher GmbH) for 6 periods of 30 s, pulse mode 0.5 and sonic power 60%. The pellet was precipitated by centrifugation (Beckman JA2-21, 10,000 g, 4 ºC, 20 min) and discarded. The supernatant was applied to a column packed with cobalt metal affinity resin and then washed three times with wash buffer (see above). NSAAR enzymes were eluted with elution buffer (100 mM NaCl, 0.02% NaN_3, 50 mM imidazole, 2 mM Tris, pH 8.0). Protein purity was determined at different stages of the purification by SDS-PAGE electrophoresis. An additional gel filtration chromatography step was carried out using a Superdex 200 gel filtration column (GE Healthcare) in a BioLogic DuoFlow FPLC system (BioRad) to eliminate any DNA co-eluting with the protein, with observation at 280 nm. The purified enzyme was concentrated using an Amicon ultrafiltration system with Amicon YM-3 membranes, dialyzed against 100 mM Borate-HCl pH 8.0 and stored at 4 ºC. Protein concentrations were determined from the absorbance of coefficient extinction of tyrosine residues [39].

Standard Enzymatic assay

A standard enzymatic reaction was carried out to assess the activity of GstNSAAR, DrcNSAAR and GkaNSAAR enzymes toward different *N*-substituted-amino acids. Purified enzymes (0.1-50 µM) were incubated together with different *N*-substituted-amino acids (15 mM), using 100 mM Borate/HCl buffer 1.6 mM $CoCl_2$ pH 8.0 (500 µl reaction volume). The reaction mixture was incubated at 45 ºC, and aliquots of 75 µl were retrieved at different times and boiled at 95 ºC for 5 min to stop the enzymatic

reaction. Then, 675 µl of the corresponding mobile phase (see below) was added to the stopped sample before centrifugation (13,000 rpm, 10 min). Samples were analyzed in a HPLC system (LC2000Plus HPLC System, Jasco) equipped with a Chirobiotic T column (4.6 mm x 250 mm, ASTEC Inc., USA). The mobile phase was 70% methanol, 30% ammonium acetate (0.01 M), and 0.5 ml acetic acid per liter [33], pumped at a flow rate of 0.6 $ml \cdot min^{-1}$ and measured at 200 nm. The specific activity of the enzymes was defined as the amount of enzyme that catalyzed the formation of 1 mmol of *N*-D- or *N*-L-substituted amino acid per min and mg of protein at 45 ºC.

Characterization of GstNSAAR enzyme

In order to get the apo-form of GstNSAAR, freshly purified enzyme (2.3 µM) was incubated overnight with 25 mM HQSA, followed by extensive dialysis in 100 mM Borate-HCl buffer pH 8.0. To analyze the effect of different cations on GstNSAAR activity, apo-GstNSAAR was incubated separately in the presence of 2 mM NaCl, KCl, LiCl, CsCl, RbCl, $MgCl_2$, $CaCl_2$, $ZnCl_2$, $CoCl_2$, $NiCl_2$, $FeCl_2$, $PbCl_2$, $HgCl_2$ in 100 mM Borate-HCl buffer pH 8.0 (final volume 20 µl) at 4 ºC for 60 minutes, followed by the standard enzyme assay. The standard enzyme assay was used to determine optimum temperature and pH of GstNSAAR. The temperature range was 20 to 85 ºC and the buffers used were 100 mM sodium citrate (pH 4.0-6.0), 100 mM sodium phosphate (pH 6.0-8.0), 100 mM sodium Borate-HCl (pH 8.0-9.0), Tris-HCl (pH 7.5-9.0) and 100 mM Borate-NaOH (pH 9.0-10.5). Thermal stability of the enzyme (2.3 µM) in 100 mM Borate-HCl buffer pH 8.0 was determined after 60-min preincubation at different temperatures from 20 to 85 ºC in the absence or presence (1.6 mM) of $CoCl_2$, followed by the standard activity assay. Kinetic studies of GstNSAAR were conducted using different *N*-formyl-D- and *N*-formyl-L-amino acids as substrate in 100 mM Borate-HCl

buffer 1.6 mM $CoCl_2$ pH 8.0 following the standard assay, with concentrations of substrate up to 50 mM. The activity of GstNSAAR mutants was measured using the standard activity assay described above, increasing enzyme concentration up to 50 µM.

Alanine scanning of GstNSAAR K166, D191, E216, D241 and K265

Mutagenesis was performed using QuikChange II Site-directed mutagenesis kit from Stratagene following the manufacturer's protocol, using the pET22b+ plasmid containing GstNSAAR as template. Mutations were confirmed by using the dye dideoxy nucleotide sequencing method in an ABI 377 DNA Sequencer (Applied Biosytems). The plasmids containing the mutated residues (K166A, D191A, E216A, D241A, K265A), were transformed into *E. coli* BL21 (DE3) and protein overexpression and purification were carried out as described above for the wild-type enzyme.

Circular Dichroism experiments

The secondary structures of GstNSAAR mutants (K166A, D191A, E216A, D241A and K265A) were compared to the wild-type GstNSAAR using far-UV circular dichroism (CD) spectra, recorded with a Jasco J850 CD spectrometer (Jasco Inc.) equipped with a JASCO PTC-423S/15 Peltier accessory. Experiments were acquired with a response time of 8 s, a bandwidth of 1 and a step resolution of 0.2 nm. Protein concentrations were 2-6 µM in 10 mM Borate-HCl buffer pH 8.0. CD measurements were taken at 25 ºC using a 1-mm path-length cuvette. Spectra were acquired from 250 to 190 nm at a scan rate of 50 $nm \cdot min^{-1}$, and averaged over 5 scans.

For thermal denaturation experiments, CD spectra were measured in 100 mM Borate-HCl buffer 1.6 mM $CoCl_2$ pH 8.0 at a protein concentration of 5 µM in a 0.1 mm

cuvette. Thermal denaturation measurements were monitored by measuring the changes in ellipticity at 222 nm. Denaturation data were collected at a scan rate of 0.2 ºC·min^{-1} and the temperature was increased from 25 to 95 ºC.

Binding experiments with GstNSAAR mutants

Fluorescence emission spectra were measured at 25 °C using an FP-6500 spectrofluorimeter (Jasco Inc.) equipped with an ETC 273T Peltier accessory with the proper excitation and emission wavelengths, using a cell of 1 cm path length. Enzymes were excited at 280 nm in order to obtain the intrinsic fluorescence spectra. The binding of *N*-formyl-D- or *N*-formyl-L-methionine to alanine mutants of GstNSAAR was monitored using the decrease in fluorescence emission at 341 nm. Excitation and emission bandwidths were 3 nm. Fluorescence measurements were corrected for dilution.

The saturation fraction, Y, can be expressed as:

$$Y = \frac{K[\text{Ligand}]}{1 + K[\text{Ligand}]} \quad (1)$$

where K is the characteristic microscopic association constant and [Ligand] is the free concentration of *N*-formyl-D- or *N*-formyl-L-methionine. The saturation fraction, Y, can be calculated as:

$$Y = \frac{\Delta F_{corr}}{\Delta F_{corr}^{max}} = \frac{F(\text{Ligand}) - F(0)}{F(\infty) - F(0)} \quad (2)$$

where F(0), F(Ligand) and F(∞) are the corrected fluorescence intensities for the protein solution without ligand, at a concentration of ligand equal to *N*-formyl-D- or *N*-formyl-L-methionine and at saturating ligand concentration, respectively.

Results and Discussion

Sequence analysis of GstNSAAR and DrcNSAAR

A BLASTn search with the nucelotide sequence of *Geobacillus stearothermophilus* CECT49 revealed 99% identity with the locus tag GK0926 from *Geobacillus kaustophilus* HTA426 (GenBank acc. No BA000043, region 949470-950594; prot BAD75211.1) and 97% identity with the previously isolated *N*-succinyl-amino acid racemase gene from *Geobacillus kaustophilus* CECT4264 (EU427322.1). On the other hand, the BLASTn search with the nucelotide sequence of *Deinococcus radiodurans* CECT833 NSAAR genes revealed 100% identity with the locus tag DR_0044 from *Deinococcus radiodurans* R1 (GenBank acc. No AE000513, region 42775-43899; prot AAF09631.1). The translated sequences of both genes were used to carry out a sequence alignment with the other NSAAR enzymes described in the literature for which the sequence is available (Figure 2). As expected, the higher sequence identities of GstNSAAR and DrcNSAAR were found with those of the other NSAAR from the same genera (GstNSAAR and GkaNSAAR, 97.6% seq. id.; DrcNSAAR and *Deinococcus radiodurans* CCRC 12827 (DraNSAAR, [8]); 98.7% seq. id.)). Similar results were obtained with other NSAAR from the same genera (*Amycolatopsis orientalis* subsp. *lurida* and *Amycolatopsis azurea*; 95.1% seq. id.). The *Geobacillus* and *Deinococcus* NSAARs shared a 47-48% of seq. id. Sequence identities of GstNSAAR and DrcNSAAR with the other NSAAR from *Amycolaptosis* and *Thermus* were 42.8-48.5% and 43.6-59.7%, respectively. A recent work already highlighted that NSAARs cannot be easily segregated into a family separate from the OSBS family, due to the high levels of sequence similarity and the bifunctionality shown by several of some OSBS/NSAAR family enzymes [40]. This fact would clearly explain why only the activity of NSAARs from five different microbial genera (see above) has been

described in the literature (decreasing to three cases for which sequence-activity relationship has been proved; *Geobacillus*, *Deinococcus* and *Amycolaptosis*). Eight subfamilies into the OSBS family of enzymes have been differentiated [40]; one of them is the so-called *Firmicutes* OSBS/NSAR subfamily, where the enzymes with proven NSAAR activity (*Geobacillus*, *Deinococcus* and *Amycolaptosis*) and that from *Thermus* are included. However, this subfamily also contains OSBS enzymes without NSAAR activity, such as *Bacillus subtilis* YtfD, sharing more than 40% of sequence identity with NSAAR of proven activity [10]. Thus, a BLAST search strategy intended to look for new real NSAAR enzymes with potential biotechnological application might not be the most successful strategy, even if a high sequence identity cut-off is used, since as denoted above, sequences showing 40-50% sequence identity do not always encode enzymes with NSAAR activity.

Expression and purification of NSAAR enzymes

Purification of GstNSAAR and DrcNSAAR enzymes yielded 5-20 mg of protein *per* liter of the recombinant *E. coli* culture. SDS-PAGE analysis indicated that the different enzymes were over 95% pure after elution of the affinity column (Figure 3), with an estimated molecular mass of 43 kDa (the deduced mass from the amino acid sequences, including the His_6-tag, is in the 43-44 kDa range). SEC-experiments conducted on a Superdex 200 10/300 column showed that GstNSAAR eluted at 15.2 mL (Figure 3), corresponding to an apparent molecular mass of 184±5 kDa. Since the theoretical molecular mass of a GstNSAAR tetramer is 174.3 kDa, our results suggests a tetrameric species (or a compacted higher-order oligomer). Similar results were obtained previously with GkaNSAAR (170-177 kDa) [31]. The NSAAR from *Steptomyces atratus* Y-53 (SatNSAAR) was described as an hexamer [3], whereas the enzymes

belonging to *Amycolaptosis* [4,6,7] and *Deinococcus* [8] genera have been reported as octamers.

Characterization of GstNSAAR enzyme

Activity of GstNSAAR was assayed in the presence of different metal cations (Table 1). All the tested cations increased the activity of the apoenzyme, although cobalt exerted the highest increase. GstNSAAR enzyme was also active after IMAC purification (43.2±0.4% relative activity compared to the Co-amended enzyme), most likely due to the NaCl present in the elution buffer, or even to Co^{2+} leakage from the IMAC column used for purification. Similar results have been shown with other NSAAR enzymes [3-9], although Mg^{2+} has been reported as the normal divalent metal ion utilized by members of the enolase superfamily [41]. Thus, all the characterization of GstNSAAR was carried out in the presence of this cofactor. GstNSAAR showed maximum activity at pH 8.0-8.5 and 55-65 ºC. Similar results have been shown for other NSAAR enzymes ranging pHs 7.5-8.0 and 40-60 ºC [3-9]. Thermal stability of GstNSAAR was studied by means of two techniques: 1) pre-incubating the enzyme in 100 mM Borate-HCl buffer pH 8.0 at different temperatures for 60 min, and subsequently measuring the residual activity; and 2) following the denaturation melting curve by means of far-UV CD. Activity was gradually lost when the enzyme was incubated in the absence of Co^{2+} at temperatures over 45 ºC, and over 55 ºC when Co^{2+} was present during the preincubation (Figure 4). Similar results have been observed previously for GkaNSAAR, showing that the presence of the cation in the protein produces a stabilization of approximately 10 ºC [31]. In view of this decrease in activity over 55 ºC, we decided to carry out all subsequent reactions at 45 ºC.

Although the thermal denaturation followed by far-UV CD (Figure 4) was irreversible, and therefore it was not possible to estimate the thermodynamic parameters governing thermal unfolding (ΔH_{vH}), we determined an apparent thermal denaturation midpoint ($T_{m(app)}$), as has been described for other proteins showing irreversible thermal denaturations [42,43]. The $T_{m(app)}$ for GstNSAAR was 77.0±0.1 ºC (Table 3), confirming an apparent moderate thermostability of GstNSAAR, similar to that previously obtained with other NSAAR enzymes [9,31]. The differences shown between the $T_{m(app)}$ and the midpoint calculated from the residual activity after preincubation (Figure 4; approximately 15 ºC) can be explained by the Equilibrium Model [44,45]: enzymatic activity might be lowered or lost below the apparent unfolding temperature as a result of temperature-induced conformational changes at the active site from an optimum configuration for substrate binding to a less optimum one. Previous results with GkaNSAAR showing a different optimal temperature for *N*-formyl- and *N*-carbamoyl-amino acid racemization [32] also support temperature-induced conformational changes of the catalytic center.

Substrate specificity of GstNSAAR

Whereas the major efforts on the activity characterization of NSAAR/NAAR enzymes have been focused on their *N*-acetyl-racemase activity, NSAAR/NAAR enzymes belonging to *Streptomyces*, *Amycolatopsis*, *Deinococcus* and *Geobacillus* have also been reported to racemize to very different extents other *N*-substituted-amino acids such as *N*-propionyl-, *N*-butyryl-, *N*-benzoyl-, *N*-acetyl-, *N*-chloroacetyl-, *N*-carbamoyl-, *N*-succiniyl- and *N*-formyl-amino acids [3,4,6,7,9,16,32,33,35]. *Streptomyces* and *Amycolatopsis* NAARs are able to racemize *N*-formyl-amino acids (more slowly than the acylated species) [3,4], but GkaNSAAR was the first NSAAR enzyme reported to

racemize *N*-formyl-amino acids much faster than *N*-acetyl- or *N*-carbamoyl-amino acids [9]. In order to compare the catalytic properties of GstNSAAR and DrcNSAAR enzymes with those of previously characterized NSAAR enzymes, we also purified GkaNSAAR enzyme [9]. The enzymes were firstly confirmed to be active towards *N*-succinyl-D- and L-phenylalanine (Table 2). GstNSAAR and DrcNSAAR were also active to different degrees toward *N*-acetyl-, *N*-carbamoyl- and *N*-formyl-amino acids (Table 2). Under the reaction conditions, GstNSAAR and GkaNSAAR were more efficient in all cases than DrcNSAAR (Table 2). Interestingly, although DraNSAAR has been mainly characterized and used for its *N*-acetyl- and *N*-carbamoyl-racemase activities [8,29,30,34], our results show that DrcNSAAR racemizes *N*-formyl-methionine faster than the *N*-acetyl- and *N*-carbamoyl-derivatives, as observed with GstNSAAR and GkaNSAAR. Whereas GkaNSAAR has been the first NSAAR enzyme successfully used in the DKR of *N*-formyl-amino acids [32,33], kinetic studies with *N*-formyl-amino acids had not been conducted previously [9]. Thus, kinetic parameters were obtained from hyperbolic saturation curves by least-squares fit of the data to the Michaelis-Menten equation (Figure 5A). Reactions were carried out with different *N*-formyl-amino acids at varying concentrations (1-50 mM) at 45 ºC and pH 8.0. The best substrate among those tested was *N*-formyl-homophenylalanine, with both the lowest K_m and highest k_{cat} values (Table 4). Taking into account the errors associated with the measurements, the first conclusion is that GstNSAAR presents a slightly higher catalytic efficiency toward the D-enantiomer of the substrates in most cases, similarly to the results observed previously with GkaNSAAR using *N*-acetyl-amino acids as substrates [9]. The k_{cat}/K_m values of GstNSAAR towards *N*-formyl-amino acids was in the 10^3-10^4 $M^{-1}\cdot s^{-1}$ range (Table 4), whereas GkaNSAAR catalytic efficiency was in the 10-10^3 $M^{-1}\cdot s^{-1}$ range with *N*-acetyl-amino acids [9]. Unexpectedly, the catalytic

efficiency of GstNSAAR towards *N*-formyl-amino acids was in the same range than the obtained for the natural substrates of *G. kaustophilus* HTA426 NSAAR (*N*-succinyl-amino acids; 10^3-10^4 $M^{-1} \cdot s^{-1}$) [16]. In fact, and whereas slightly lower, kinetic K_m and k_{cat} values of GstNSAAR towards *N*-formyl-L-methionine (9.0±0.8 mM, 52.9±1.4 s^{-1}) were very similar to those of *G. kaustophilus* HTA426 NSAAR with *N*-succinyl-L-methionine (3.6±0.4 mM, 53.0±1.0 s^{-1}) [16]. In *G. kaustophilus* HTA426, NSAAR appears in an operon together with highly enantioselective succinyl-CoA:D-amino acid *N*-succinyltransferase and *N*-succinyl-L-amino acid hydrolase enzymes, conforming a metabolic pathway for irreversible conversion of D-amino acids to their L-enantiomers [16,17]. Despite the suggested moonlight character of NSAAR [16], based on the previous results on the isolated enzymes in the *G. kaustophilus* HTA426 "succinyl-transferase/racemase/hydrolase" operon (where less than 0.1% of hydrolase activity was detected towards *N*-formyl-amino acids), the similar efficiency of GstNSAAR towards *N*-formyl- and *N*-succinyl-amino acids do not seem to support the former as an alternative natural substrate for this route.

When analyzing the *N*-formyl-amino acid racemization of GstNSAAR, GkaNSAAR and DrcNSAAR, the catalytic efficiency values using *N*-formyl-D-norleucine were 1.9±0.3, 1.7±0.3 and 0.2±0.0 $s^{-1} \cdot mM^{-1}$, respectively. Thus, *Geobacillus* enzymes presented an efficiency one order of magnitude higher than that of the *Deinococcus* enzyme, in accordance with the trend of the specific activities for the other *N*-substituted-amino acids (Table 2). Our results show that from a biotechnological point of view, even though DraNSAAR has been successfully applied in different DKR processes [29,30,34], GstNSAAR and GkaNSAAR enzymes might be better candidates

for putative industrial applications based on their higher activities with the substrates assayed (Table 2).

Effect of residues K166, D191, E216, D241 and K265 on the activity of GstNSAAR

Previous mutagenesis studies carried out with *Amycolatopsis* sp T-1-160 NSAAR already concluded that NSAAR enzymes use a two-base mechanism for racemization, more specifically two lysine residues [13]. The crystallographic structure of this enzyme (PDB ID. 1SJA) showed three additional residues involved in substrate binding [14]. The five counterpart residues of GstNSAAR are K166, D191, E216, D241 and K265. We decided to mutate these residues to alanine in order to evaluate their implication in the activity and binding of GstNSAAR. Purity of the mutants was over 95% (data not shown). Far-UV CD spectra were collected to evaluate the native-like folding of the Co-amended mutants. No significant differences were found in the far-UV CD spectra of the five alanine mutants, and we can therefore conclude that their secondary structure is basically the same as that of wild-type NSAAR (data not shown). Thermal melts were irreversible, as for the wild-type enzyme, but we determined the $T_{m(app)}$ values for all the mutants (Table 3). Since the temperature melts were irreversible, these values should be only considered as a qualitative measure of the similar stability of the WT and mutated species. Except for the thermal midpoint obtained for K265A mutant, which was higher than the rest, the melting curves, together with the steady-state far-UV data, suggest that the alanine scanning mutations did not alter significantly the native-like structure of the enzyme.

All the mutants showed negligible activity using up to 50 μM of enzyme (Table 3), confirming their key role in substrate racemization. Binding experiments with the

alanine mutants using *N*-formyl-D- and *N*-formyl-L-methionine (Figure 5B) yielded affinity constants in the range of 10^3-10^4 M^{-1} (Table 5), showing a slightly higher affinity for the D-enantiomer in all cases, and confirming these residues as not indispensable for *N*-formyl-amino acid binding despite its key role in the enzyme activity (Table 3). These results follow the same trend as the catalytic efficiency of the enzyme toward the different enantiomer pairs (Table 4), which also present a slight preference for the D-enantiomer. This slight preference agrees with the expected enantioselectivity of an NSAAR participating in the irreversible conversion of D-amino acids to their L-enantiomers using the above mentioned "succinyl-transferase/racemase/hydrolase" operon [16]. Although the genome of *G. stearothermophilus* CECT49 is not available, the one from *G. stearothermophilus* str. 53 (GenBank Acc. No. JPYV01000018) also contains the counterpart operon, suggesting that this gene organization also might be present in *G. stearothermophilus* CECT49.

Since the $T_{m(app)}$ values for the three metal-binding mutants (D191A, E216A and D241A) were very similar to that found for the wild-type NSAAR (Table 3), we could argue that the three mutants are still able to bind the cation, since the presence of Co^{2+} produced a 10 ºC increase in the thermal midpoint (see above). Whereas we cannot compare the affinity constants with those of the wild-type (they cannot be obtained as both isomers would be in solution), the affinity of the three mutants for both ligands decreased in the order D191A > D241A > E216A. By similarity of GstNSAAR and DraNSAAR (PDB ID 1XPY, [8]) the observed decrease in affinity might be a reflect of a lower polarization of the Co^{2+} cofactor by the absence of one of the carboxylate group of the lateral chains of the three amino acids, altering the interaction of the cation with the carboxylate group of the substrate. However, this cation-carboxylate interaction is

not totally indispensable for substrate binding, since we also confirmed that the three mutants are able to bind both enantiomers of the substrate, although with slightly lower affinity (data not shown). The absence of activity of the D191A, E216A and D241A mutants (Table 3) also suggest that the proposed role of stabilization of the enediolate anion intermediate of the reaction by the cofactor, is indispensable for the proposed two-base 1,1-proton transfer mechanism [8, 13]: mutation of any of the three-binding residues inactivates the enzyme, even when the cation is bound to the enzyme, hampering the racemization of the substrate.

The results with the K166A and K265A mutants are similar to those obtained previously for *Amycolatopsis* NSAAR [13]: mutation of these residues also inactivated GstNSAAR. These results are in accordance with the proposed two-base mechanism of the enolase superfamily [8,13]. It is worth noting the apparent enhancement of K265A $T_{m(app)}$ (Table 3) and the apparent higher affinity of K166A towards *N*-formyl-D-methionine (Table 5). In the absence of additional results, we could only speculate on both interesting features. The homolog DraNSAAR X-Ray structure (PDB ID 1XPY, [8]) shows that the counterpart lysine residues ($K168_{Dra}$ and $K269_{Dra}$) are situated 3.8 Å away from the cation. Since our results show that: *i)* the presence of the cation increases the GstNSAAR $T_{m(app)}$ and *ii)* mutation in the metal-binding residues and the presence of the cation alter the affinity of GstNSAAR by the substrates (Table 5), we could argue that K166A and K265A mutations might produce alterations in the environment of the Co^{2+} cation, producing two different effects. In one hand, K265A mutation might result in a tighter binding of the cofactor, resulting in an even higher stabilization of the tertiary structure of GstNSAAR, thus increasing its $T_{m(app)}$. On the other hand, since the counterpart of K166 and D191 in the DraNSAAR structure ($K168_{Dra}$ and $D195_{Dra}$; PDB

ID 1XPY, [8]) are positioned 2.8 Å away, we could argue that if K166-D191 interaction is lost due to the mutation, changes in the polarizability of the cation might occur, thus altering the affinity by the different ligands (Table 5).

Conclusion

Several studies carried out by Glasner´s group have previously highlighted two specific difficulties to find new NSAAR enzymes. Firstly, the high degree of similarity between the OSBS family [40] complicates the discovery of real NSAAR enzymes, since even members of the so-called *Firmicutes* NSAR/OSBS subfamily are not active towards *N*-succinyl-amino acids [10]. Secondly, several works have proven the difficulties to find the determinant allowing the evolution of OSBS family towards NSAAR activity [40,46,47]. The recent published studies on the role of the 20s loop of *Amycolatopsis* sp T-1-160 NSAAR [47] has shed some light on the importance of the conservation of not only the five residues mutated in this work, but other residues previously observed in the substrate-bound crystallographic structure of DraNSAAR (Figure 2) [8]. In our opinion, an alternative clue to find new biotechnologically relevant NSAAR enzymes/sequences, different to the only three for which a sequence-activity relationship has been proven (*Geobacillus*, *Deinococcus*, *Amycolaptosis*), might lie on the localization of similar *N*-succinyl-"transferase/racemase/hydrolase" operons similar to that reported previously [16].

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Table 1. Relative activity of GstNSAAR in the presence of different mono- and divalent cations, using the cobalt-amended GstNSAAR as reference. The effect of the different cations was assayed using the apo-form of GstNSAAR. Values are the mean of three experiments, and the error indicates the standard deviation of the mean.

Sample	Relative activity (%)
apo-GstNSAAR	0.0±0.0
Co^{2+}	100.0±1.1
Ni^{2+}	55.8±7.6
K^{+}	40.22±8.4
Na^{+}	42.8±10.4
Li^{+}	45.8±5.4
Cs^{+}	47.8±12.9
Rb^{+}	40.8±8.5
Mg^{2+}	28.1±2.9
Ca^{2+}	14.3±0.7
Zn^{2+}	14.4±6.5
Fe^{2+}	15.5±0.6
Hg^{2+}	3.7±4.0
Pb^{2+}	8.3±0.9

Table 2. Specific activities (μmol·min^{-1}·mg of enzyme^{-1}) of the different enzymes used in this work toward different *N*-substituted amino acids. Reaction conditions were the same in all cases for values to be comparable among them (15 mM substrate in 100 mM Borate-HCl 1.6 mM $CoCl_2$ pH 8.0, at 45 ºC)

	GKaNSAAR	**GstNSAAR**	**DrcNSAAR**
***N*-Succinyl-D-phenylalanine**	3.3±1.0	3.3±0.2	2.4±0.1
***N*-Succinyl-L-phenylalanine**	7.9±1.0	9.0±0.2	4.5±0.2
***N*-Formyl-D-methionine**	4.7±0.3	4.9±0.5	2.6±0.4
***N*-Acetyl-D-methionine**	3.6±0.0	3.7±0.0	1.6±0.0
***N*-Carbamoyl-D-methionine**	0.7±0.1	0.7±0.1	0.3±0.1
***N*-Formyl-D-norleucine**	1.7±0.3	1.9±0.3	0.2±0.0
***N*-Carbamoyl-D-norleucine**	0.3±0.0	0.3±0.0	0.1±0.0

Table 3. Thermal denaturation midpoints obtained for GstNSAAR and mutants in the presence of $CoCl_2$, and relative activities of the mutants using *N*-formyl-D- and *N*-formyl-L-methionine, compared to the wild-type enzyme.

Enzyme	Thermal denat. midpoint (º C)	Relative activity (%) *N*-formyl-D-MET	Relative activity (%) *N*-formyl-L-MET
GstNSAAR	77.0±0.1	100.00	100.00
K166A	78.5±0.1	0.02	0.01
D191A	76.5±0.1	0.00	0.02
E216A	78.7±0.1	0.01	0.01
D241A	76.9±0.1	0.01	0.02
K265A	83.4±0.6	0.01	0.04

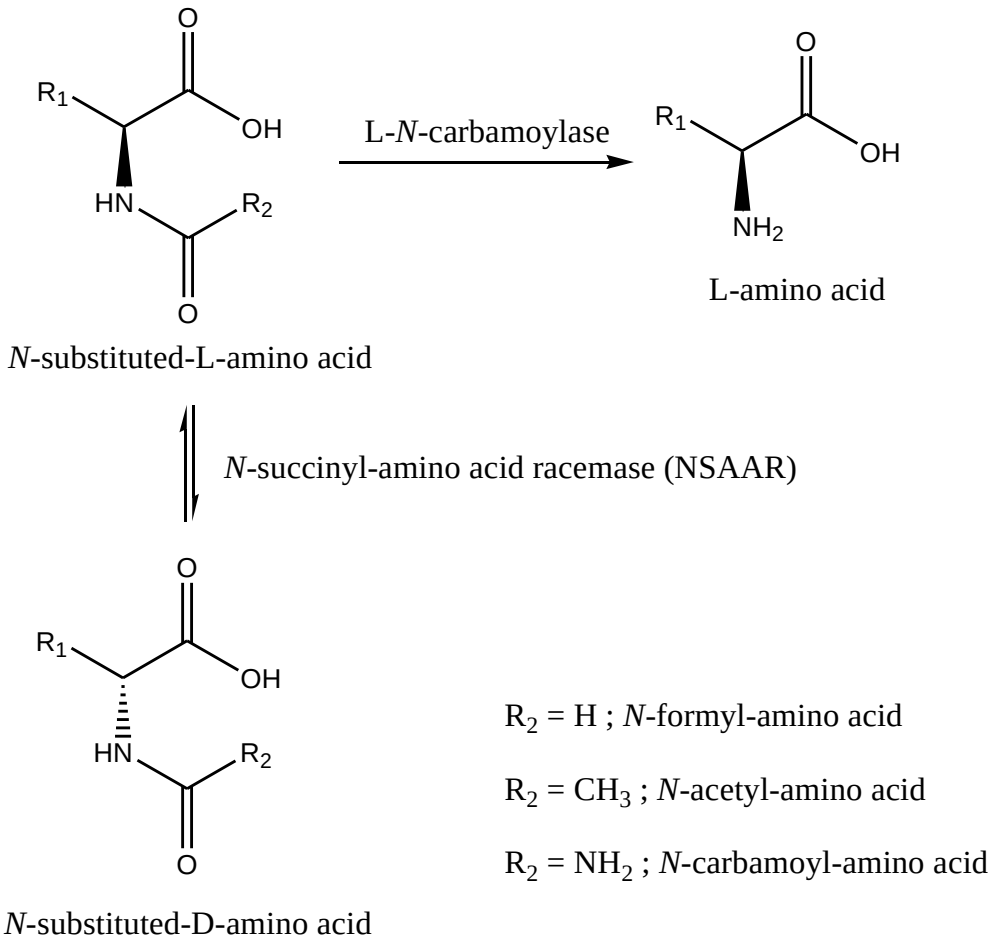
Table 4. Kinetic analyses of wild-type GstNSAAR. Kinetic parameters were obtained from hyperbolic saturation curves by least-squares fit of the data to the Michaelis-Menten equation. Reactions were carried out with the different *N*-formyl-amino acids at different concentrations (1-50 mM) at 45 ºC and pH 8.0.

Substrate	**K_m (mM)**	**k_{cat} (s^{-1})**	**k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)**
N-Formyl-D-methionine	12.6±2.7	74.6±5.1	5.9±1.7
N-Formyl-L-methionine	9.0±0.8	52.9±1.4	5.9±0.7
N-Formyl-D-norleucine	11.1±4.1	12.3±1.4	1.1±0.5
N-Formyl-L-norleucine	12.5±1.7	8.0±0.4	0.6±0.1
N-Formyl-D-aminobutyric acid	19.6±3.7	15.2±0.7	0.8±0.2
N-Formyl-L-aminobutyric acid	17.9±2.5	11.6±0.7	0.7±0.1
N-Formyl-D-norvaline	9.2±1.8	11.2±0.7	1.3±0.3
N-Formyl-L-norvaline	24.5±5.7	19.6±1.4	0.8±0.2
N-Formyl-D-homophenylalanine	3.0±0.7	88.4±4.3	29.5±8.3
N-Formyl-L-homophenylalanine	5.7±0.5	85.5±0.7	15.0±1.4

Table 5. Binding constants obtained for *N*-formyl-D- and *N*-formyl-L-methionine GstNSAAR mutants. Titration was performed at 25 ºC in 100 mM Borate-HCl 1.6 mM $CoCl_2$ pH 8.0. Enzyme concentrations were in the range 0.8-1.0 μM and were titrated by addition of different ligand volume of solution at a concentration of 50 mM of *N*-formyl-D- or *N*-formyl-L-methionine.

GstNSAAR mutant	Ligand	K (M^{-1})	Ratio D/L
K265A	*N*-Formyl-L-methionine	1017±22	1.7
	N-Formyl-D-methionine	1689±27	
D191A	*N*-Formyl-L-methionine	1871±18	1.2
	N-Formyl-D-methionine	2195±19	
D241A	*N*-Formyl-L-methionine	1444±12	1.4
	N-Formyl-D-methionine	2024±24	
E216A	*N*-Formyl-L-methionine	1113±39	1.2
	N-Formyl-D-methionine	1343±21	
K166A	*N*-Formyl-L-methionine	1792±33	2.9
	N-Formyl-D-methionine	5269±38	

Figure 1. Reaction scheme for optically pure L-amino acid production from racemic mixtures of *N*-substituted-amino acids using the "Amidohydrolase process". R_1 = lateral chain of the corresponding amino acid. R_2 = *N*-substituent. In the original "Acylase Process", R_2=CH_3.



819 **Figure 2**. Sequence alignment of the different NSAAR enzymes reported in the
820 literature. GstNSAAR, NSAAR from *Geobacillus stearothermophilus* CECT49 (this
821 work); GkaNSAAR, NSAAR from *Geobacillus kaustophilus* CECT4264, GenBank
822 accession no. ABZ81711 [31]; DrcNSAAR, NSAAR from *Deinococcus radiodurans*
823 CECT833 (this work); DraNSAAR, NSAAR from *Deinococcus radiodurans*, GenBank
824 accession no. 1R0M [8]; AspTS160, NSAAR from *Amycolatopsis* sp. TS-1-60,
825 GenBank accession no. BAA06400 [4,5]; Aorlur, NSAAR from *Amycolatopsis*
826 *orientalis* subsp. *lurida*, GenBank accession no. CAC00653 [6]; Aaz, NSAAR from
827 *Amycolatopsis azurea*, GenBank accession no. AF335269_1 [7]; Thermus, putative
828 NSAAR from *Thermus termophilus*, accession No BAD70697.1 [34]. The residues
829 predicted to most likely configure the catalytic pocket of DraNSAAR are highly
830 conserved among the different NSAAR above described, and are shown under the
831 alignment with letters following the original nomenclature [8] (C: carboxyl group-
832 binding site; M: metal-binding site; L1 and L2: L region; S: side-chain-binding region).
833

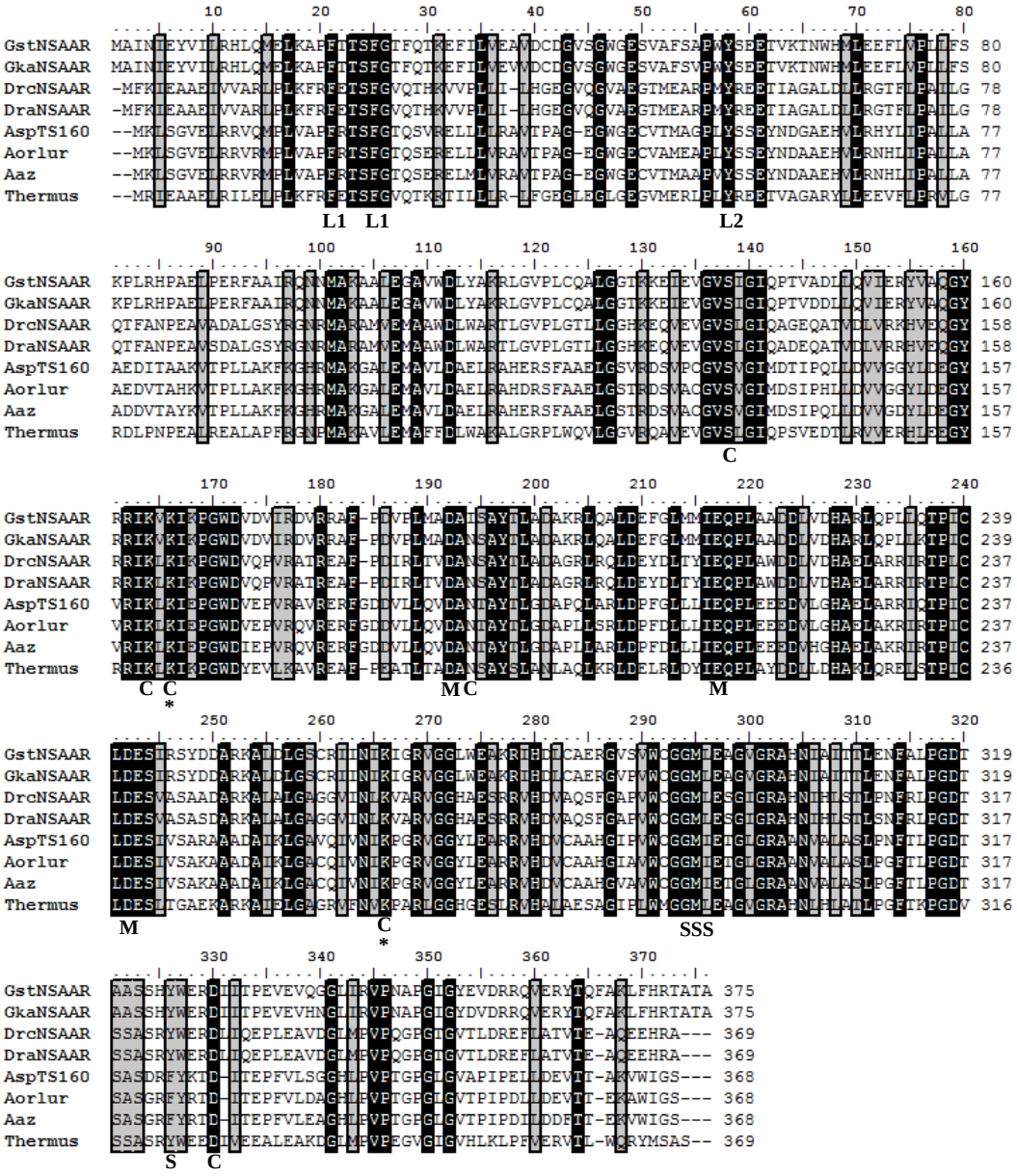

GstNSAAR
GkaNSAAR
DrcNSAAR
DraNSAAR
AspTS160
Aorlur
Aaz
Thermus
L1 L1
L2
C
C C
*
M C
M
M
C
*
SSS
S C

Figure 3. Size exclusion chromatography elution profile of GstNSAAR using a Superdex 200 10/300 column. The inset represents the SDS-PAGE of the different purified recombinant NSAAR enzymes. Lane 1, low molecular weight marker; lanes 2, 3, and 4, purified GstNSAAR, GkaNSAAR, and DrcNSAAR, respectively.

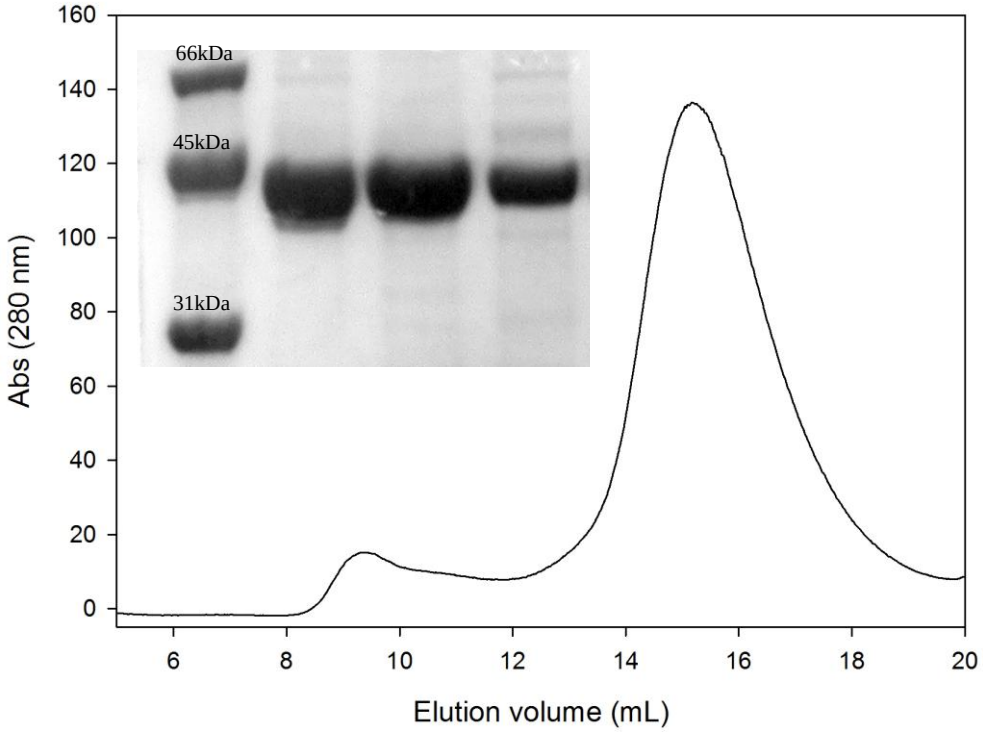


Figure 4. CD-Thermal denaturation of cobalt amended GstNSAAR followed by the changes in ellipticity at 222 nm (continuous line, right axis). The plot also shows the remaining relative activity of GstNSAAR after 60-min preincubation at the corresponding temperature, both in the presence (●) and absence (○) of Co^{2+} (left axis). The results showing the remaining activity of GstNSAAR are the mean of three experiments, and the error bars indicate the standard deviation of the mean.

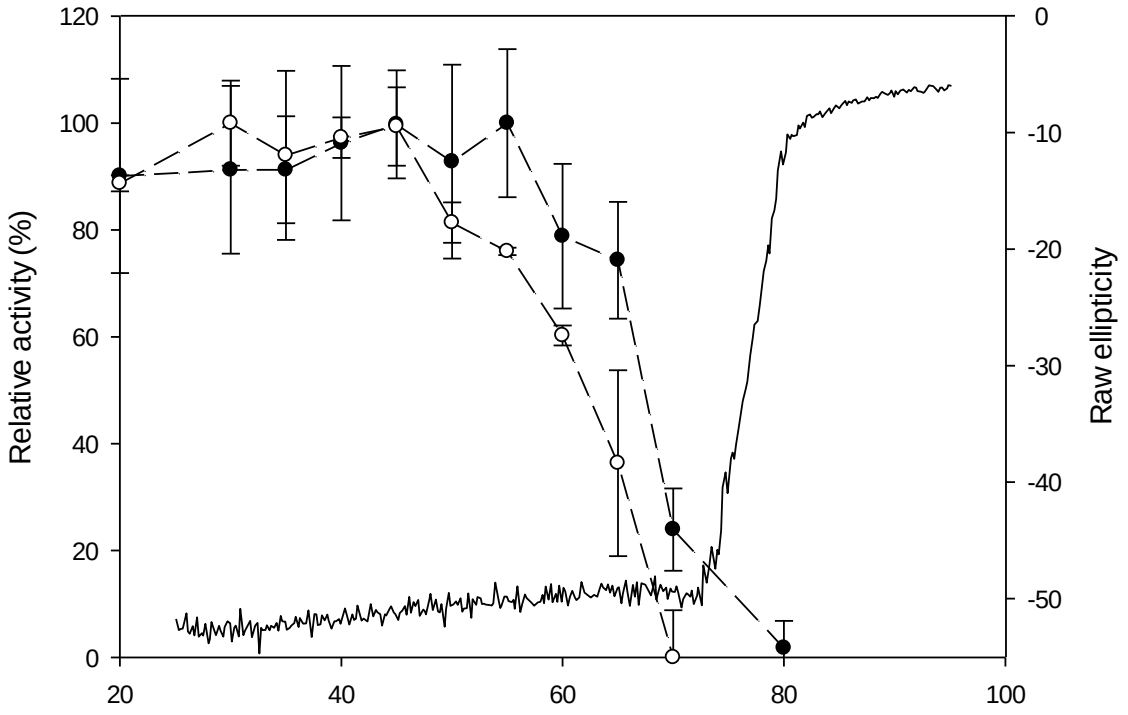


Figure 5. A) Kinetics of GstNSAAR using *N*-formyl-L-homophenylalanine as substrate (45 ºC and pH 8.0). The results are the mean of three experiments, and the error bars indicate the standard deviation of the mean. B) Fluorescence titration of *N*-formyl-D-methionine binding to E216A and K166A GstNSAAR mutants. Titrations were performed in 100 mM Borate-HCl buffer pH 8.0 and 25 °C, with enzyme concentrations in the range 0.81-0.95 μM. Stock ligand concentrations were 50 mM.