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Tyrosine binding and promiscuity in the arginine repressor from the pathogenic bacterium *Corynebacterium pseudotuberculosis*

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ABSTRACT

The arginine repressor (ArgR) regulates arginine biosynthesis in a number of microorganisms and consists of two domains interlinked by a short peptide; the N-terminal domain is involved in DNA binding and the C-terminal domain binds arginine and forms a hexamer made-up of a dimer of trimers. The crystal structure of the C-terminal domain of ArgR from the pathogenic *Corynebacterium pseudotuberculosis* determined at 1.9 Å resolution contains a tightly bound tyrosine at the arginine-binding site indicating hitherto unobserved promiscuity. Structural analysis of the binding pocket displays clear molecular adaptations to accommodate tyrosine binding suggesting the possible existence of an alternative regulatory process in this pathogenic bacterium.

1. Introduction

Arginine is synthesized in prokaryotes from glutamate in eight enzymatic steps [1] and transcription in this pathway is negatively regulated by the arginine repressor (ArgR) [2]. At specific concentrations of arginine, ArgR oligomerizes and binds to the operator DNA related to the arginine metabolic pathway preventing its interaction with RNA polymerase by steric hindrance thereby interrupting arginine synthesis [3]. The bacterial mechanism of regulation of arginine biosynthesis, the amino acid sequence of ArgR and the nucleotide sequence of its cognate operator sites display a high degree of conservation [4–7]. Each subunit of ArgR consists of two domains interlinked by a short peptide; the N-terminal domain is involved in DNA binding and the C-terminal domain binds arginine and forms a hexamer made-up of a dimer of trimers. Six arginines bind at the subunit interfaces inducing a rotation of one trimer with respect to the other, thus enhancing the DNA-ArgR interactions. In the structures of ArgR from *Mycobacterium tuberculosis* [8], *Escherichia coli* [9,10] and *Bacillus stearothermophilus* [11], this rotation is 11, 13 and 15°, respectively and is stabilized by the interaction of the highly conserved Asp136 [12] with the guanidinium groups of the bound arginines [11].

Although several features of this mechanism are conserved in diverse organisms, ArgR's from the hyperthermophiles *Thermotoga neapolitana* function via a mechanism that is atypically independent of the arginine concentration [13]. In the case of *Corynebacterium glutamicum*, a very efficient producer of amino acids and hence used industrially, the expression of the Arg operon is not influenced by the arginine concentration [14]. However, upon proline supplementation, the ArgR-binding affinity is significantly reduced upstream of argB, suggesting that proline may act as an anti-repressor by binding to a specific region of ArgR [15]. *Streptomyces clavilugerus,* widely used in the industrial production of clavulanic acid [16,17], utilizes arginine as a specific precursor [18] and ArgR has been reported to be involved in the regulation of the production of this molecule [19]. During fermentation, succinyl L-tyrosine, an undesired by-product of clavulanic acid, is produced [16].

We determined the crystal structure of the C-terminal domain of ArgR from *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis*) at 1.9 Å resolution with a tyrosine bound at the putative arginine-binding site. This binding pocket displays

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clear molecular adaptations to accommodate tyrosine suggesting the possible existence of a different regulatory process in this pathogenic bacterium and indicating its biological relevance.

2. Material and methods

2.1. Protein expression and purification

Escherichia coli C43 (DE3) pLysS transformed with the expression vector pD441containing the gene corresponding to ArgR protein of *C. pseudotuberculosis* was used for expression. The product of expression includes an N-terminal hexahistidine tag and a TEV cleavage site (ENLYFQG). An isolated colony of *E. coli* was grown for 16 h at 37 °C in LB medium supplemented with kanamycin (34 ug/mL). The culture was then diluted 100-fold with fresh LB broth containing kanamycin (34 ug/mL) and incubated at 30 °C until the optical density (OD_{600}) reached 0.5 AU and was subsequently induced with 0.2 mM IPTG for 6 h at 30 °C. The cells were harvested by centrifugation at 5000 g for 10 min at 4 °C and resuspended in a 20 mM Tris-HCl buffer, pH 8.0, containing 300 mM NaCl. The clear supernatant obtained by centrifugation at 15,000 g for 30 min was subjected to affinity chromatography using an immobilized nickel column (GE) under native conditions and a second step of ion-exchange chromatography was performed using a Mono Q 5/50 GL (GE Healthcare Life Sciences) column and the fraction containing ArgR was concentrated and further purified using a Superdex G 75 10/300 GL column. All purification steps were analyzed by 15% SDS-PAGE gels [20].

2.2. Crystallization, data collection and processing

Crystals were obtained by the hanging drop vapor diffusion method by mixing 1 μL of the protein sample at 8 mg mL^{-1} in 10 mM Tris-HCl buffer pH 7.5 and 100 mM NaCl with an equal volume of the reservoir solution (100 mM Tris-HCl, pH 7.5, 200 mM NaCl and 10% ethanol). The crystals grew in 20 days with approximate dimensions of 100 × 100 × 200 μm. For data collection, a single ArgR crystal was directly flash-cooled in a 100 K nitrogen-gas stream and X-ray diffraction data were collected on the W01B-MX2 beamline at the Brazilian Synchrotron Light Laboratory (Campinas, Brazil). The wavelength of the radiation source was set to 1.458 Å and a Pilatus 2 M detector was used to record the diffraction intensities. The data were indexed, integrated and scaled using the XDS Package [21].

2.3. Structure determination, refinement and validation

The structure was solved by molecular replacement using the atomic coordinates of ArgR from *Mycobacterium tuberculosis* (PDB ID: 3ERE, 51% sequence identity) and the program PHASER [22]. Model refinement was carried out using cycles of REFMAC5 [23] followed by visual inspection of the electron density maps and manual rebuilding with COOT [25]. The final model was validated using the MOLPROBITY server [24]. Data collection and refinement statistics are presented in Table 1. The ArgR atomic coordinates have been deposited with the RCSB Protein Data Bank under the accession code 5JVO.

3. Results and discussion

3.1. The C-terminal domain of C. pseudotuberculosis ArgR binds tyrosine

The *C. pseudotuberculosis* ArgR full-length protein was expressed, purified and crystallized. Two different crystal forms were observed in the same crystallization drop, one form diffracted only to 5 Å and the second form, diffracted to 1.9 Å and was used for structure determination. The structure was determined by molecular replacement using the atomic coordinates of the enzyme from *Mycobacterium tuberculosis* (PDB ID 3ERE, sequence identity 51%) and refined to crystallographic residuals of 0.15/0.19 (*R*/*R*free) (Table 1). The asymmetric unit contains two molecules of the C-terminal domain (residues 82–160), suggesting cleavage in the linker region between the N- and C-domains by trace amounts of endogenous proteases as reported earlier [26,27]. Each subunit of the C-terminal domain of the ArgR chain exhibits a α/β topology in which the three α-helices alternate with a pair of strands resulting in a αββαββα fold (Fig. 1A). The crystal symmetry operations in space group $P2_13$ result in the formation of a hexamer made up of a dimer of trimers (Fig. 1B), which is stable in solution according to PISA (Protein Interfaces, Surfaces, and Assemblies) analysis [28]. This hexameric arrangement is conserved in ArgRs [29–36] and it is proposed that arginine binding to this oligomer has a stabilizing effect on the proper conformation required for the binding of this transcriptional repressor to its DNA target sequence in the upstream region of the operon [37–41]. Interestingly, the Arg-binding site in *C. pseudotuberculosis* ArgR is occupied by a tyrosine that is very clearly defined in the electron density map (Fig. 1A–C). Analysis of the biological hexamer reconstructed by symmetry operations indicated that six tyrosines were present in the highly acidic pocket which is conserved in all ArgR homologues (Fig. 1B). Moreover, the two trimers are related to each other by a relative rotation of 15.8°, indicating that tyrosine binding had the same effect as arginine binding in inducing a conformational change that is required for optimal DNA binding thus indicating the relevance of tyrosine binding.

Table 1
Data collection and refinement statistics.

Wavelength (Å)	1.458
Resolution range (Å)	48.00–1.90 (2.01–1.90)[a]
Space group	P 2_13
Unit cell parameters (Å)	a = b = c = 83.09
Total reflections	160,590 (18,725)
Unique reflections	15,299 (2429)
Multiplicity	10.5 (7.7)
Completeness (%)	99.9 (99.5)
Mean I/sigma (I)	20.81 (2.15)
R-merge	0.079 (0.899)
R-meas	0.083 (0.965)
$CC_{1/2}$	0.99 (0.69)
R-work/R-free (%)	0.179/0.224
Number of protein chains in A.U.	2
Ligand molecules	3 (2 tyrosine/1 SO_4)
Solvent water molecules	49
RMSD (bonds) (Å)	0.026
RMSD (angles) (°)	2.372
Ramachandran analysis	
Favored regions (%)	99.3
Allowed regions (%)	0.7
Outliers (%)	0
Average B-factor	31.13
PDB accession code	5JVO

[a] Statistics for the highest-resolution shell are presented in parentheses.

3.2. Structural determinants for tyrosine binding

The Arg/Tyr binding pocket located at the interface of the trimer involves the participation of residues from subunits A, B and A' (symmetry equivalent molecule) (Fig. 1B). The interactions with the amino and carboxyl groups of both ligands, tyrosine (Fig. 2A) and arginine (Fig. 2B), are fully conserved. The amino group is anchored to the residues Asp122, Thr132, Asp137 and Thr138, whereas the

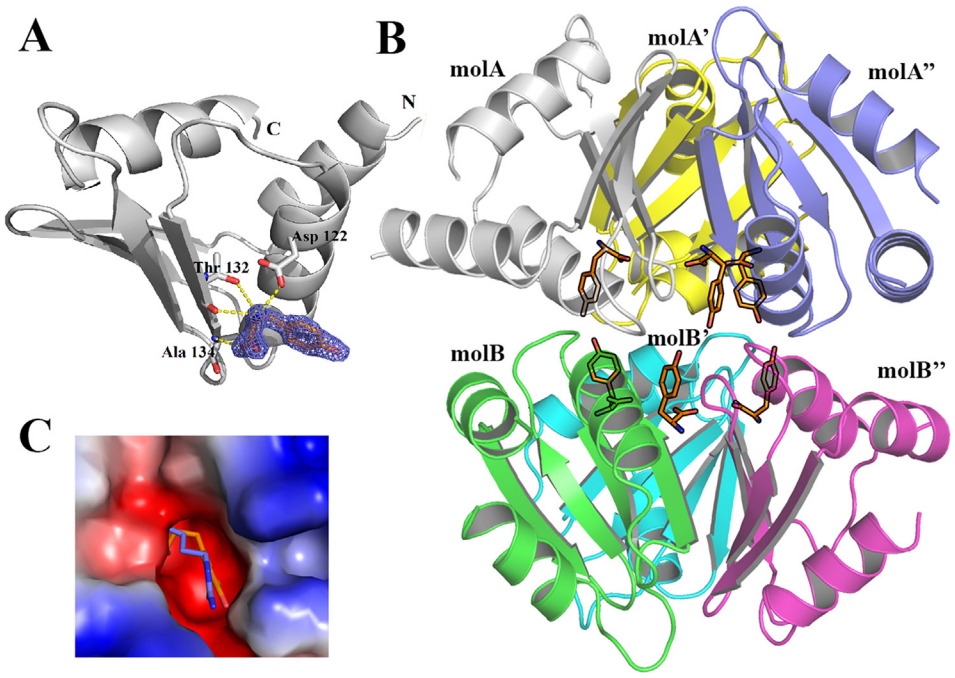

Fig. 1. (A) Ribbon representation of the three-dimensional structure of the C-terminal domain of ArgR, blue cage represents the 'omit' map electron density of the bound arginine (contoured at 2.0 σ amino acids that form hydrogen bonds (yellow) to tyrosine are included. (B) Hexameric arrangement of ArgR with bound tyrosines (orange). (C) Super positioning of arginine (blue) and tyrosine (orange) and charge distribution in the Arg/Tyr binding pocket. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

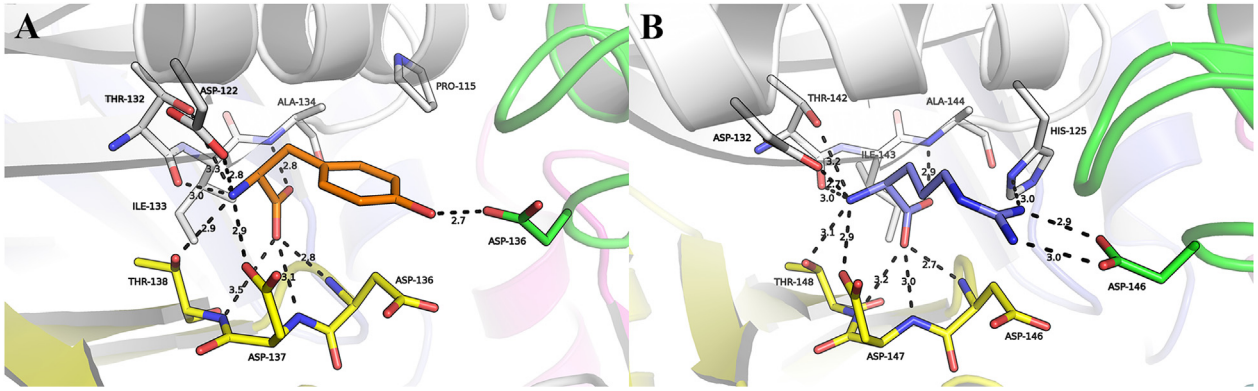

Fig. 2. Hydrogen bond interactions with the bound ligands (tyrosine in orange and arginine in blue) and the residues of ArgR hexamers of (A) *C. pseudotuberculosis* and (B) *M. tuberculosis*. ArgR residues; carbon atoms in yellow and green represent residues from symmetry related molecules. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

carboxyl group is hydrogen bonded to the main-chain amide groups of the residues Ala134, Asp136 and Asp137 (Fig 2A–B) (amino acid numbering based on the sequence of ArgR from *C. pseudotuberculosis*). These residues are conserved in the ArgR structures from *B. stearothermophilus*, *E. coli*, *M. tuberculosis* and *V. vulnificus* except for *Bacillus subtilis* which contains a cystine at position 134 (PDB IDs:1B4B, 1XXA, 2ZFZ, 3V4G and 2P5M respectively). In the presence of an arginine, the guanidinium group forms an electrostatic interaction with Asp136 (equivalent to Asp146 in PDB 2ZFZ) (Fig. 2B). Tyrosine forms a hydrogen bond with the side-chain atom, OD2, of Asp136 (Fig. 2A).

Comparative structural analysis indicates that all key residues involved in the binding of either ligand are strictly conserved, suggesting that other substitutions are involved in the selection of either arginine or tyrosine as ligands (Fig. 3A). Examination of the region in the neighborhood of the ligand pocket revealed that an invariant polar residue, His or Gln is generally present at position 115 (Table 2, Fig. 3) and interacts with the guanidinium group of arginine (Figs. 2B and 3). In *C. pseudotuberculosis* ArgR, this position is occupied by a proline, which is less bulky and has the ability to form hydrogen bonds (Figs. 2A and 3). This substitution is the most likely determinant for *C. pseudotuberculosis* ArgR accepting tyrosine as a ligand, since the presence of a large polar residue at this position would promote severe steric impediments for tyrosine binding (Fig. 3). The presence of a proline residue creates a room for the accommodation of the phenol group of the tyrosine ligand and also supports formation of aliphatic and Van der Waals contacts. However, it is worth mentioning that this modification does not preclude arginine recognition and binding, indicating that either amino acid could function as a high affinity ligand.

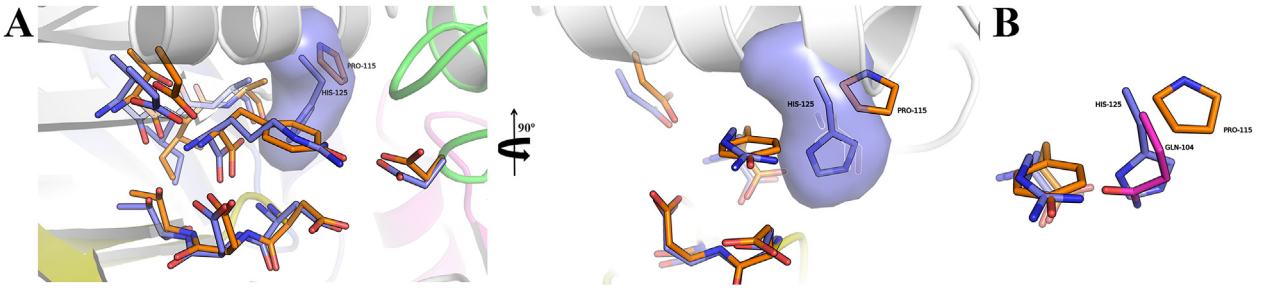

Fig. 3. Effect of amino acid substitutions in the Arg/Tyr binding pocket of *C. pseudotuberculosis* (orange); *M. tuberculosis* (blue) and *B. subtilis* (pink); (A) Substitution of His (surface volume in blue) by Pro better accommodates Tyr (orange). (B) Effect of substitution of Pro115 by Gln/His. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Overall sequence identity, domain rotation and sequence in the arginine-binding pocket of ArgRs: Blue = position corresponding to Pro115 in *C. pseudotuberculosis*, which is either Gln or His in all Arg-ArgR structures deposited with the PDB; underlined residues = residues that form hydrogen bonds with arginine/tyrosine; red = totally conserved residues.

Organism	PDB ID	Sequence identity (%)	Ligand	Rotation	Arginine-binding pocket residues 115#/122/132/134/136-138
M. tuberculosis	2ZFZ	54	R	11°	PGAAHYLASAIDRAALPQ-VVGTIAGDDT
B. stearothermophilus	1B4B	38	R	15°	PGNAHAIGVLLDNLDWDE-IVGTICGDDT
B. subtilis	2P5M	35	R	15°	PGNAQAIGALMDNLDWDE-MMGTICGDDT
V. vulnificus	3V4G	31	NO LIGAND	-	PGAAQLIARMLDSLGKSEGILGVVAGDDT
E. coli	1XXA	37	R	13°	PGAAQLIARLLDSLGKAEGILGTIAGDDT
C. pseudotuberculosis	5JVO	-	Y	15.8°	PGGAPFLASFIDRVGMEE-VVGTIAGDDT

3.3. ArgR promiscuity and the putative role in regulation

Protein interactome studies have identified proteins that participate in exceptionally large numbers of interactions and multi-specificity is observed for many proteins involved in signaling and regulation [42]. Except for the structure of ArgR from *M. tuberculosis* (PDB: 3LAP) bound to L-canavanine (*non-proteinaceous* ligand structurally related to L-arginine), we present the first ArgR structure with a non-arginine ligand occupying the putative arginine-binding site.

Analysis of the genome of *C. pseudotuberculosis* revealed the presence of a gene encoding a Tyrosyl-tRNA synthetase immediately down-stream to the arginine biosynthesis gene cluster (Fig. 4). It permits us to speculate on the possible role of the complex ArgR-Tyr in regulating this synthetase, since in *Enterococcus durans* the tyrosyl-tRNA synthetase like gene is regulated by tyrosine concentration [43].

ArgR promiscuity can assist improving our understanding of diverse regulatory mechanisms that have not yet been elucidated such as in *T. neapolitana*, where the binding of ArgR to DNA is independent of the arginine concentration [13]. The *C. glutamicum* Arg operon is not influenced by arginine [14], but the ArgR-binding affinity is significantly reduced upstream of argB upon proline supplementation [15]. The ArgR-Pro complex may be involved in Arg and/or Pro regulation based on the promiscuity of the binding pocket.

The knowledge that tyrosine can bind and stabilize the ArgR complex of *Corynebacterium tuberculosis* along with studies that demonstrate that *Streptomyces clavilugerus* uses ornithine and arginine as specific precursors of clavulanic acid (CA) [18] may provide insights about other functions of these molecules. The involvement of ArgR in regulation of CA production has been reported [44,45], and during this process succinyl L-tyrosine, is formed as a by-product. The regulation of CA synthesis is not fully elucidated, however, a putative ARG box, which is characteristic of genes regulated by ArgR, has been identified in the oat2 gene

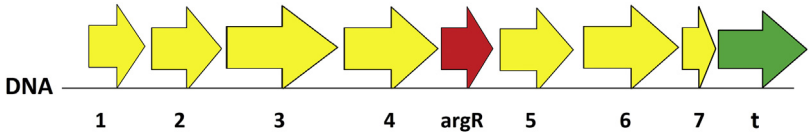

Fig. 4. Gene cluster of *arginine biosynthesis*; Glutamate *N*-acetyltransferase (1), Acetylglutamate kinase (2), Acetylornithine aminotransferase (3), Ornithine carbamoyltransferase (4), Argininosuccinate synthase (5), Argininosuccinase lyase (6), Hypothetical protein (7). In yellow genes involved in the transformation of glutamate into arginine; argR gene (red) and the tyrosyl-tRNA synthetase gene (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

present in the CA gene cluster of *Streptomyces clavuligerus*. The argJ gene present in the arginine gene cluster shares high similarity with the oat2 gene, and the purified *S. clavuligerus* ArgR protein is reported to bind to the ARG box in the oat2 promoter [45] and its expression is negatively controlled by ArgR [46]. Succinyl L-tyrosine was modeled into the arginine-binding pocked of *C. pseudotuberculosis* which suggests a possible regulatory role in CA synthesis and needs to be confirmed experimentally.

Our results indicate that amino acid substitutions at position 115 determine whether tyrosine may serve as a putative ligand and may modulate ArgRs. Since the arginine biosynthesis pathway is present in the pathogen and is absent in the host, analogues of tyrosine that induce oligomerization of ArgR and stimulate DNA binding and hence suppress arginine synthesis, make the promiscuity of ArgR an attractive target for drug design.

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