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Crystal structure of the Aspartyl-tRNA synthetase from *Entamoeba histolytica*

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Abstract

The crystal structure of the aspartyl-tRNA synthetase from the eukaryotic parasite *Entamoeba histolytica* has been determined at 2.8 Å resolution. Relative to homologous sequences, the *E. histolytica* protein contains a 43-residue insertion between the N-terminal anticodon binding domain and the C-terminal catalytic domain. The present structure reveals that this insertion extends an arm of the hinge region that has previously been shown to mediate interaction of aspartyl-tRNA synthetase with the cognate tRNA D-stem. Modeling indicates that this *Entamoeba*-specific insertion is likely to increase the interaction surface with the cognate tRNA^{Asp}. In doing so it may substitute functionally for an RNA-binding motif located in N-terminal extensions found in AspRS sequences from lower eukaryotes but absent in *Entamoeba*. The *E. histolytica* AspRS structure shows a well-ordered N-terminus that contributes to the AspRS dimer interface.

Keywords

tRNA ligase

1. Introduction

Aminoacyl-tRNA synthetases mediate the key biological function of translating an RNA message into a corresponding protein sequence. Each specific aminoacyl-tRNA synthetase is responsible for attaching a particular amino acid to its proper cognate tRNA. In general this results in a charged tRNA that uniquely associates the anticodon sequence it carries on its anticodon loop with the amino acid it carries on its acceptor stem. A few exceptional cases require subsequent additional chemical modification of the attached amino acid. For example, the aspartyl-tRNA synthetase found in many bacteria and archaea does not discriminate between tRNA^{Asp} and tRNA^{Asn}, charging both with aspartate [1]. The mis-charged Asp-tRNA^{Asn} is then converted to Asn-tRNA^{Asn} by a separate amidotransferase. As expected for

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a molecule with such a key role, the aminoacyl-tRNA synthetases are found in all living organisms. They group into two classes defined by sequence phylogeny and by the consequent structural homology of their core domains. The Class I catalytic domain contains a Rossmann fold and two highly conserved sequence motifs. The Class II catalytic domain contains a core antiparallel β -sheet surrounded by α -helices, and is uniquely identified by the presence of three highly conserved sequence motifs not found in Class I. Further subdivision into subclasses a, b, and c is based on additional regions of homology. The synthetase for each amino acid except lysine belongs consistently to either Class I or Class II across all forms of life. Despite this broad conservation of core structural and functional features, many individual aaRS have evolved additional inserted domains, idiosyncratic sequence variation, and even whole new biological functions on top of their already essential role. This combination of core essentiality and individual variation suggests that individual aaRS may be useful targets for the development of new drugs for infectious disease [review: 2].

Entamoeba histolytica is a food- and water-borne pathogen estimated to infect 50 million people annually. It is a major cause of dysentery and has the potential to cause life-threatening abscesses in the liver and other organs. Entamoebiasis is prevalent throughout developing nations and estimated to cause more than 100,000 deaths per year. As is the case with other eukaryotic parasites, the few existing drugs have significant side-effects. The potential emergence of drug-resistance to current chemotherapeutics is also a continuing concern. The aspartyl-tRNA synthetase (AspRS) from *E. histolytica* was chosen for study by the Medical Structural Genomics of Pathogenic Protozoa (MSGPP) collaborative as part of a broader effort to investigate aminoacyl-tRNA synthetases as potential targets for drug discovery [3]. The *E. histolytica* genome codes for only a single AspRS. Unlike some higher eukaryotes, and indeed unlike some other protozoans, the *Entamoeba* genome does not contain a gene for a separate mitochondrial AspRS related to bacterial homologs. The *E. histolytica* AspRS sequence groups phylogenetically with the cytosolic AspRS sequences from other eukaryotes. It is closest to trypanosomatid sequences, which split from archaeal sequences earlier than the divergence of plant, fungal, and other higher eukaryotic lineages [4]. AspRS sequences from lower eukaryotes have idiosyncratic differences from those of higher eukaryotes. For example, *Trypanosoma brucei* has evolved distinct cytosolic and mitochondrial isoforms of AspRS, both derived, presumably by gene duplication, from a eukaryotic cytosolic homolog [4]. AspRS sequences from *Plasmodia* contain several plasmodia-specific insertions, including a ~30 residue insertion in the first half of the N-terminal domain which appears to be required for proper dimerization [5]. The *E. histolytica* AspRS contains a 43 residue insertion between the N- and C-terminal domains. This is seen in the present crystal structure to extend the arm containing helix $\alpha 6$, which mediates binding of the cognate tRNA^{Asp}. Given the essential nature of aminoacyl-tRNA synthetases in general, these idiosyncratic features are of interest as possible targets for anti-protozoan drug design.

We report here a crystal structure of the apo AspRS from *E. histolytica* determined at 2.8 Å resolution. Crystal structures have been reported for one other eukaryotic AspRS, the yeast cytosolic homolog [6,7,8], as have structures from two archaeal homologs [9,10], and several more distantly related bacterial homologs. The *E. histolytica* AspRS sequence is 47% identical overall to that of the yeast and human homologs, but differs from them by the presence of the *Entamoeba*-specific hinge region insertion and by at least two amino acid substitutions among the active site residues involved in substrate binding [Figure 1].

2. Materials and Methods

The full length sequence corresponding to GenBank accession no. [EAL44507.1](#), coding for *E. histolytica* AspRS, was cloned from cDNA into the AVA0421 vector and expressed in *E. coli*. Protein was purified on a Ni-NTA affinity column followed by cleavage of the N-terminal

affinity tag. Purified protein consisted of 4 residues remaining from the cleavable tag followed by the 544 residues of AspRS. Initial crystallization conditions found by the high throughput crystallization screening laboratory at the Hauptman-Woodward Institute [11] were later optimized to yield data-quality crystals. Crystals used for data collection were grown by vapor diffusion from sitting drops equilibrated against a reservoir containing 0.2 M lithium sulfate, 0.1 M bis-Tris buffer at pH 5.5, and 23% w/v PEG 3350. The initial crystallization drops consisted of 0.2 μ l/protein at 9 mg/ml in standard buffer [12] plus 0.2 μ l of the reservoir solution.

Crystallographic data to 2.8 Å resolution were collected from a single crystal at beamline 11-1 of the Stanford Synchrotron Radiation Lightsource. The structure was determined by automated molecular replacement using BALBES [13] followed by manual correction of sequence alignment errors relative to the search probe fragments that BALBES had generated from PDB entries 1eov, 1b8a, 1wyd, 1x54, 1n9w, and 1q46. Crystallographic refinement proceeded using alternating cycles of refinement in Refmac5 [14] and model building in Coot [15]. The refined crystallographic model included a correction for partial merohedral twinning and a multi-segment model for atomic displacements generated by TLSMD [16]. The final model has been deposited in the Protein Data Bank with accession code **3i7f**. Structural superpositions were performed using Coot and SSM [17].

3. Results and Discussion

3.1. Conserved structural features

AspRS is a Class Iib aminoacyl-tRNA synthetase: it is a homodimer; its catalytic domain has a 6-strand antiparallel β -sheet at its core; it contains a subclass-specific anticodon recognition domain. The *E. histolytica* AspRS crystal structure contains one dimer in the asymmetric unit of the crystal. Both monomers of the dimer are well-ordered except for nine N-terminal residues and the regions comprised of residues 178–208 and 269–287.

Residues 26–163 form the characteristic Class Iib anticodon recognition domain. The *E. histolytica* AspRS is 38% identical in sequence to the yeast cytosolic AspRS in this domain. The N-terminal domain from the current structure can be superimposed onto that of the apo yeast AspRS structure (PDB entry 1eov) with an RMSD of 1.1 Å for 138 C^α atoms, leaving 4 gaps where the yeast sequence contains inserted residues relative to both the *E. histolytica* and human AspRS homologs.

Residues 233–544 form the Class II aminoacyl-tRNA synthetase catalytic core domain. The overall sequence identity between the *E. histolytica* and yeast sequences in this domain is 62%. The backbones of the two apo structures diverge in the region of residues 384–385, remote from both the active site and the tRNA interaction surfaces. Here the yeast sequence has a 2-residue insertion relative to the *E. histolytica* or human AspRS homologs. A second divergence is observed at residues 317–329 (equivalent to yeast residues 325–337) where the conformation of the current apo *E. histolytica* AspRS structure is essentially the same as that of the tRNA-bound yeast structure (1asy) rather than that of the apo yeast structure (1eov). Indeed, the overall conformation of the catalytic domain in the current structure superimposes more closely onto the yeast tRNA-bound complex (RMSD of 1.0 Å for 293 C^α atoms, with 3 gaps) than it does onto the yeast apo structure (RMSD of 1.2 Å for 285 C^α atoms, with 6 gaps). However, in the region where the apo and tRNA-bound yeast structures are maximally different, corresponding to residues 269–278 in *E. histolytica*, the current structure is not well-ordered. This region includes the 'flipping loop', which has been observed to vary between open and closed conformations depending upon the presence of aspartate, aspartyladenylate, and/or the tRNA acceptor arm [18].

3.2. N-terminal extension

Structural homology among the yeast, archaeal, and *E. histolytica* proteins begins at the equivalent of *E. histolytica* residue 26 (yeast residue 73). Residues N-terminal to this point constitute an N-terminal extension to the conserved anticodon binding domain whose length varies in AspRS homologs from different organisms. These N-terminal extensions have acquired distinct biological functions, although this region was not observed as an ordered domain in previous crystal structures of AspRS. The N-terminal extension of most eukaryotic AspRS contains a short, lysine-rich, RNA-binding motif that increases affinity for tRNA. Footprinting experiments show that in yeast cytosolic AspRS this N-terminal extension contacts the tRNA anticodon stem [19]. If the yeast enzyme is modified by deletion of the N-terminal 70 residues, including the RNA-binding motif, it retains full activity and the ability to dimerize, but exhibits a 100-fold loss in affinity (K_M) for *tRNA*^{Asp} [19]. Mutation of the equivalent RNA-binding motif in *P. falciparum* AspRS impairs both K_M and k_{cat} [5].

In distinction to this, AspRS sequences from mammals and birds are exceptional in having a shorter (~25 residue) N-terminal extension that does not contain a recognizable RNA-binding motif [5]. The N-terminus of mammalian AspRS has been characterized as contributing to the association of AspRS with a multi-enzyme complex containing multiple aminoacyl-tRNA synthetases [20]. NMR studies of a synthetic peptide corresponding to residues 5–25 of human AspRS show that in isolation the initial residues adopt a flexible conformation containing a β -turn, while residues 16–23 form a regular α -helix [21]. The immobilized peptide interacts weakly with tRNA [21].

The short N-terminal extension in the *E. histolytica* AspRS is similar in length to that of mammalian homologs rather to the longer extensions of other protozoa. It does not contain a recognizable RNA-binding motif. As observed in the present crystal structure, the *E. histolytica* AspRS N-terminus is well-ordered beginning at residue 10. It assumes an extended conformation that is stabilized by interaction of residues 20–26 with helix $\alpha 4$ in the same monomer, and by the contributions of residues 11–17 to formation of the dimer interface through interaction with residues 493–503 of the second monomer. This yields a dimer interface with a total buried solvent-accessible surface of 4650 Å² per monomer [22], somewhat larger than that seen for the yeast or archaeal AspRS homologs but smaller than that found in the *Thermus thermophilus* AspRS (1efw). Thus a substantial structural rearrangement, including partial disruption of the dimer interface, would be required in order for the N-terminal extension of the *E. histolytica* enzyme either to adopt the helical conformation observed by NMR for the human peptide or to interact directly with bound tRNA. While such rearrangement may be possible, it seems more probable that this functional role is instead assumed by a separate *Entamoeba*-specific insertion as discussed below.

3.3. Extended arm in the hinge region

Between the anticodon binding domain and the catalytic core domain of AspRS is a hinge region that plays a key role in binding *tRNA*^{Asp} [23]. In previous structures this hinge region has been observed to form three short helices, $\alpha 6$ – $\alpha 8$ in Figure 1, that interact directly with the backbone ribose and phosphate groups of U11 and U12 on the tRNA D-stem and also with the tRNA acceptor arm [23]. The *E. histolytica* AspRS sequence is atypical in that this hinge region contains an additional 43 residues (Figure 1). A BLAST search against the NCBI sequence database found no homologous insertion outside of the genus *Entamoeba*. In the current structure, 12 residues of this insertion are observed to form a well-ordered extension of helix $\alpha 6$ and of the paired non-helical stretch linking it to helix $\alpha 7$ (Figure 2a). The result of this is that the arm containing helix $\alpha 6$ protrudes at least 15 Å further from the bulk of the protein than it does in the homologous yeast structure. Furthermore, although the electron density beyond this point is not clear enough to fully model the remaining 31 residues of the

insertion, it suggests that the arm continues as an ordered structure beyond residue 177. This region of the sequence is strongly predicted to be helical by Jpred [24].

The angle of the extended arm relative to the N- and C-terminal domains is different in the two crystal-lographically independent monomers of the current structure. This is consistent with the 'hinge' role played by this region, which pivots to mediate the binding of tRNA and its subsequent repositioning relative to the active site [7]. Superposition onto the yeast AspRS:tRNA^{Asp} complex suggests that the observed extension of the $\alpha 6$ arm in *E. histolytica* AspRS is likely to increase the interaction surface with tRNA, and hence may contribute to tRNA binding by interacting with additional residues in the D-stem (Figure 2b). It is interesting to speculate that this interaction may substitute functionally for the RNA-binding motif observed in the long N-terminal extension of other eukaryotic AspRS sequences. This speculation is supported by the observation that the *E. histolytica* insertion sequence, like the RNA-binding motif, is a lysine-rich region with helical character.

3.4. AspRS active site is an unattractive drug target

AspRS must perform two sequential reactions. The first reaction is the formation of aspartyladenylate from the free amino acid and ATP, releasing pyrophosphate. The second reaction is the displacement of the adenylate moiety by the 3'-OH of the terminal adenosine of the tRNA^{Asp} acceptor arm, yielding the covalently linked Asp-tRNA^{Asp}. The active site must therefore bind the two initial reactants, Asp and ATP, and also provide access for the properly positioned acceptor stem of a bound tRNA molecule. Interference with either of these reactions would be sufficient to disrupt the biological function of the enzyme. As our motivation is the identification of potential opportunities for the development of anti-parasitic drugs, a key goal is to identify differences between the parasite and human enzymes that might allow selective interference.

The residues involved in Asp and ATP binding are strongly conserved across AspRS sequences from all forms of life, including the bacterial/mitochondrial branches of the phylogenetic tree. Only two of the residues expected to interact directly with these substrates differ significantly between the *E. histolytica* enzyme and its two human homologs (Figure 1). Thr469 in the current structure corresponds to a leucine in the human and yeast cytosolic AspRS homologs, and to a glycine in the more distantly related human mitochondrial homolog. The peptide backbone in this region forms part of the ATP binding site; the backbone carbonyl of the adjacent residue was observed to provide a hydrogen bonding partner for O3' of the ATP ribose in the homologous yeast AspRS:ATP complex (1asz). However the sidechain of this residue points away from the bound ATP, and the backbone conformations of the current *E. histolytica* structure and of the previously reported yeast homologs are essentially identical. Therefore this sequence difference does not appear to provide any leverage for the design of a selective inhibitor.

Similarly, residue Cys512 in the current structure corresponds to a glycine in the human and yeast cytosolic homologs and to an isoleucine in the human mitochondrial enzyme. This residue is adjacent to the aspartyl carboxylate of bound aspartyladenylate, but again the sidechain points away from the bound ligand and the backbone conformation seen in the *E. histolytica* and yeast structures (PDB 1il2) are essentially the same.

3.5. Prospects for selectively targeting *E. histolytica* AspRS

Thus analysis of the aspartate and ATP binding regions of the active site seen in the current *E. histolytica* AspRS structure does not reveal any easily exploitable structural features that might lead to design of inhibitors selective for the parasite enzyme over the human homolog. This is not promising, as the inhibitors of AspRS reported to date are all analogs of

aspartyladenylate, including the natural product antibiotic Microcin C, which is metabolized to a modified aspartyladenylate containing an N-acylphosphoramidate linkage [25]. Nor are there obvious exploitable differences adjoining the substrate binding sites. For example, one previously reported crystal structure of an archaeal AspRS (1wyd) captured a molecule of 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) bound at the position of the AMP adenine, with the sulfate moiety extending deeper into the binding pocket, but there are no useful sequence differences between the parasite and human enzymes in this region either.

An alternative approach is conceivable, via design of a compound that either exerts an allosteric effect on the active site interferes with tRNA recognition, binding, or orientation in the active site. This possibility is exemplified by the allosteric inhibition of ThrRS, another Class II aaRS, by the natural product Borrelidin, which is selectively active against the malarial parasite [26]. The current structure reveals that the presence of an *Entamoeba*-specific sequence insertion in the AspRS hinge region leads to a significant structural difference in a helix, $\alpha 6$, that interacts with the tRNA D-stem. Since this region has previously been characterized as mediating presentation of the acceptor arm to the active site, it may be possible to selectively interfere with the second reaction carried out by the enzyme. For instance, a small molecule binding to this area of the enzyme might reduce the affinity for tRNA. Or it might lead to mispositioning of the terminal adenosine of the acceptor arm within the active site, leading to a nonproductive state. This remains speculation, however, and the *E. histolytica* AspRS does not appear to constitute an easy target for the development of selective anti-parasitic drugs. It does, however, offer a view of a second eukaryotic AspRS structure, and thus may be compared with the well-studied yeast homolog to provide insight into organism-specific structural variation within the context of an otherwise highly conserved structural framework.

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Figure 1.

Structure-based sequence alignment of cytosolic AspRS from *E. histolytica* (residues 10–544), yeast (residues 57–557), and human (residues 11–501). Secondary structure is shown for the current structure. Conserved features including the three signature motifs characteristic of all Class II aaRS are indicated in blue. An *Entamoeba*-specific 43-residue insertion extends the hinge region between the N-terminal anti-codon binding domain and the conserved Class II aaRS catalytic core domain. Sequence differences at the active site are indicated by red boxes.

Figure 2.

(a) *E. histolytica* AspRS dimer. One monomer is colored from blue (N-terminal) to red (C-terminal). The second monomer is in grey. The three helices ($\alpha 6$, $\alpha 7$, $\alpha 8$) of the hinge region between the N-terminal anticodon binding domain and the C-terminal Class II aaRS catalytic domain are separately labelled. The two monomers are crystallographically independent, but are essentially identical in conformation except for the angle at which an extended arm protrudes from the hinge region. The arms (highlighted by grey circles) are more than 15 Å longer than the homologous structures of yeast AspRS, with the extension formed by an *Entamoeba*-specific 43 residue insertion. The active site is indicated by placement of a model for aspartyladenylate (blue CPK spheres) based on superposition of the binding mode seen in a bacterial AspRS:aspartyladenylate complex (PDB entry 1c0a).

(b) Model of tRNA binding to *E. histolytica* AspRS. Superposition of tRNA^{Asp} (gold surface) from the Yeast AspRS:tRNA complex (PDB 1asy) onto the current structure of *E. histolytica* AspRS (monomer B in dark blue, monomer A in light blue). The tRNA interacts only with one monomer. The 12 well-ordered residues from the *Entamoeba*-specific insertion are shown in magenta; 30 additional residues are not well-ordered in the crystal structure and are represented by dots.

Table 1

Crystallographic data collection and refinement statistics

Data collection	
Wavelength (Å)	0.97945
Resolution (Å)	124-2.8 (2.9-2.8)
Space group	<i>H</i> 3
Unit cell (Å)	a=247.8 b=247.8 c=56.3 $\alpha=90^\circ$ $\beta=90^\circ$ $\gamma=120^\circ$
Unique reflections	31,605 (3,062)
R_{merge}	0.100 (0.735)
Completeness (%)	99.7 (97.6)
Mean $I/\sigma(I)$	18.9 (1.9)
Redundancy	6.2 (4.8)
Wilson B factor (Å ²)	77
Refinement	
Resolution (Å)	124-2.8
R / R_{free}	0.200 / 0.237
standard uncertainty (s.u.) of bond lengths (Å)	0.012
standard uncertainty (s.u.) of bond angles (°)	1.4
Mean B_{eq} protein (Å ²)	65.3
TLS groups	
Chain A: 10-170, 171-225, 226-268, 289-335, 336-544	
Chain B: 10-167, 168-224, 225-268, 289-338, 339-544	
Twinning fraction	0.15
Residues with ϕ, ψ in most favored regions (%)	95.0
Residues with ϕ, ψ in allowed regions (%)	4.6
Residues with ϕ, ψ in disfavored regions (residues)	4