Crystal structure of aspartyl-tRNA synthetase from *Pyrococcus kodakaraensis* KOD: archaeon specificity and catalytic mechanism of adenylate formation

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The crystal structure of aspartyl-tRNA synthetase (AspRS) from *Pyrococcus kodakaraensis* was solved at 1.9 Å resolution. The sequence and three-dimensional structure of the catalytic domain are highly homologous to those of eukaryotic AspRSs. In contrast, the N-terminal domain, whose function is to bind the tRNA anticodon, is more similar to that of eubacterial enzymes. Its structure explains the unique property of archaeal AspRSs of accommodating both tRNA^{Asp} and tRNA^{Asn}. Soaking the apo-enzyme crystals with ATP and aspartic acid both separately and together allows the adenylate formation to be followed. Due to the asymmetry of the dimeric enzyme in the crystalline state, different steps of the reaction could be visualized within the same crystal. Four different states of the aspartic acid activation reaction could thus be characterized, revealing the functional correlation of the observed conformational changes. The binding of the amino acid substrate induces movement of two invariant loops which secure the position of the peptidyl moiety for adenylate formation. An unambiguous spatial and functional assignment of three magnesium ion cofactors can be made. This study shows the important role of residues present in both archaeal and eukaryotic AspRSs, but absent from the eubacterial enzymes.

Keywords: archaeon/aspartyl-tRNA synthetase/crystal structure/*Pyrococcus*

Introduction

Aminoacyl-tRNA synthetases catalyse the specific esterification of a given amino acid to the 3' end of its corresponding tRNA through a two-step reaction. During the first step, the amino acid and ATP substrates are converted, in the presence of Mg²⁺, into a reactive aminoacyl adenylate. In the second step, this activated amino acid is transferred to the 3' end of the cognate

tRNA. This two-step mechanism is conserved in all aminoacyl-tRNA synthetases. Analysis of the primary sequences as well as determination of the three-dimensional structures allowed the partition of the aminoacyl-tRNA synthetases into two classes (Eriani *et al.*, 1990) characterized by two different structural solutions for their active site domain and a different first site of aminoacylation. The active site of the class I enzymes is built around a Rossmann fold, based on a parallel β -sheet. Two short peptidic sequences, HIGH and KMSKS, are markers of such an organization. The catalytic centre of class II enzymes is built around an antiparallel β -sheet and characterized by three conserved motifs (motifs 1, 2 and 3).

We report the first structure of an aminoacyl-tRNA synthetase from an archaeon, AspRS from the hyperthermophilic organism, *Pyrococcus kodakaraensis* KOD1. The existence of the Archaea raises many intriguing evolutionary questions (Doolittle, 1996). These organisms seem to have transcriptional and translational apparatus close to those seen in eukaryotes, whereas the biochemical pathways resemble those seen in bacteria. AspRS synthetase is particularly well suited for investigating this aspect at the molecular level since the three-dimensional structures of both bacterial and eukaryotic representatives are known (Ruff *et al.*, 1991; Delarue *et al.*, 1994). In agreement with the correlation made from sequence analysis (Imanaka *et al.*, 1995), this crystal structure shows that its catalytic domain involves residues that are found in eukaryotic AspRSs but not in those of bacteria. In contrast, the N-terminal domain responsible for the specific recognition of the tRNA anticodon loop is closer to that of bacteria. Furthermore, its structure exhibits specific features, which explains the degenerated specificity of this archaeal enzyme which can charge both tRNA^{Asp} and tRNA^{Asn} in agreement with the absence of AsnRS in the corresponding genomes (Curnow *et al.*, 1996).

The crystal structure of a class II ternary complex between AspRS and its cognate tRNA in the presence of the aminoacyl adenylate in the yeast system provided the first data at near atomic resolution for the two steps of the aminoacylation reaction, the activation of the amino acid and its transfer to the 3' end of the tRNA (Ruff *et al.*, 1991; Cavarelli *et al.*, 1994). In the aspartic system, the first step of the aminoacylation reaction can occur in the absence of the tRNA; it is thus possible to study the two steps of the reaction separately. The first observation of a class II enzyme with an aminoacyl adenylate formed *in situ* was made using the *Thermus thermophilus* enzyme (Poterszman *et al.*, 1994). The binding of ATP, amino acids and/or adenylate analogues was also analysed in a few other systems (Belrhali *et al.*, 1994, 1995; Arnez *et al.*, 1995, 1997; Onesti *et al.*, 1995; Cusack *et al.*, 1996; Arnez and Moras, 1997). However, up to now it

Table I. Data collection and refinement statistics

	Native	ATP-Mg ²⁺ + aspartate	ATP-Mn ²⁺	Aspartic acid
Data set				
Resolution (Å)	15–2.4	15–1.9	15–1.9	15–1.95
Completeness ^a (%)	94.7 (88.0)	96.0 (86.0)	95.9 (73.4)	98.7 (82.7)
Redundancy	5.7	3.8	3.7	3.6
No. of unique reflections	51036	103073	105560	98902
R_{sym} (I) (%) ^{a,b}	9.9 (21.0)	5.0 (29.3)	5.0 (26.9)	4.8 (21.6)
Refinement				
R -factor (%)	21.1	18.1	16.8	20.4
Working set	45455	94876	95958	89636
R_{free} (%)	26.5	20.5	20.2	23.6
Test set	2543	5028	5102	4754
Water molecules	92	965	1230	408
R.m.s.d.				
bond lengths (Å)	0.008	0.011	0.013	0.007
angles (°)	1.53	1.56	1.70	1.36
dihedrals (°)	24.4	23.1	23.2	23.1
impropers (°)	1.17	0.97	1.04	1.1
R.m.s.d. between monomers (C _α atoms only)	0.58	0.82	0.63	0.62
Average B -factor (Å ²)				
enzyme	38.1/37.4	17.2/17.3	16.7/17.1	21.3/20.9
water	34.2	30.3	31.0	26.6
substrate		13.5/19.8 ^c	19.3/16.9	22.6
metallic ions ^d				
monomer 1			19.6/39.0/31.8	
monomer 2		30.2/37.6/33.1	18.6/39.8/28.6	

The native data set was collected at ESRF (Beamline D2AM), diffraction data of the complexes were measured at LURE (Orsay, France).

^aThe values in parentheses correspond to the highest resolution shell.

$$^b R_{\text{sym}} (I) = \frac{\sum_{hkl} \sum_i | \langle I_{hkl} \rangle - I_{hkl,i} |}{\sum_{hkl} \sum_i I_{hkl,i}} \quad \text{where } i \text{ is the number of reflection } hkl.$$

^cAverage B -factor for aspartyl adenylate in monomer 1 and ATP in monomer 2.

^dIsotropic B -factor for the three metallic ions: Mg1, Mg2 and Mg3 in the case of the ATP-Mg²⁺ + aspartate data set, Mn1, Mn2 and Mn3 in the case of the ATP-Mn²⁺.

was never possible to obtain, for a given synthetase, all the complexes corresponding to the course of the aminoacyl adenylate synthesis reaction. In this study, four different structural states of AspRS were characterized at atomic resolution. The structure of the apo-enzyme, and of the enzyme complexed with ATP in the presence of magnesium or manganese, with the aspartic acid and with the aspartyl adenylate were solved. Structural analysis of these complexes provides a high resolution image for the various components of the reaction in their active state and allows the role of the catalytic residues, the metal ions and the mobile elements of the enzyme active site to be defined precisely.

Results

Structure determination

The crystal structure of the native enzyme was solved initially by molecular replacement using a polyaniline model derived from the C-terminal catalytic domains of the dimeric *Saccharomyces cerevisiae* enzyme (Ruff *et al.*, 1988; Cavarelli *et al.*, 1993, 1994). From the initial positioning of the two 2-fold related domains in the asymmetric unit, an electron density map was calculated which allowed the precise location of the two N-terminal anticodon-binding domains. The N- and C-terminal domains of each monomer were then linked and, after a rigid body refinement, the model could be built and refined to an R -factor of 21.1% at 2.4 Å resolution using standard

Table II. Comparison of the pkAspRS between its different binding states

	Aspartic acid	Aspartyl adenylate	ATP
Overall (Å) (438 C _α)			
Apo enzyme	0.35	0.60	0.38
Aspartic acid		0.44	0.45
Aspartyl adenylate			0.64
Catalytic domain (308 C _α)			
Apo enzyme	0.39	0.50	0.42
Aspartic acid		0.31	0.52
Aspartyl adenylate			0.56
Anticodon domain (110 C _α)			
Apo enzyme	0.21	0.27	0.22
Aspartic acid		0.17	0.15
Aspartyl adenylate			0.17

The r.m.s. values were obtained after superimposition of the C_α atoms corresponding to monomer 1 of each structure.

methods (see Materials and methods and Table I for the summary of the crystallographic data). The crystal structures of the various complexes were all obtained from native protein crystals soaked in mother liquors containing the substrates. Subsequent refinements led to R -factors of between 17 and 20% at 1.9 Å resolution (Table 1).

Overall description of the apo-enzyme

The dimeric pkAspRS structure (2×438 amino acids) is shown in Figure 1A. Like all known three-dimensional

Fig. 1. (A) Ribbon representation of the dimeric aspartyl-tRNA synthetase from *P. kodakaraensis* KOD1 complexed with aspartyl adenylate in monomer 1 and ATP-Mg²⁺ in monomer 2. The N-terminal domain of the enzyme is coloured in yellow, the hinge region is in green, the C-terminal domain is in cyan and the flipping loop is in red. Aspartyl adenylate and ATP-Mg²⁺ are drawn in the ball and stick representation. The figure was generated using the programs MOLSCRIPT (Kraulis, 1991) and raster3D (Bacon and Anderson, 1988). (B) Schematic representation of the topology of pkAspRS. The β -strands are represented as arrows and the helices as rods. Secondary structure elements were assigned by using the PROCHECK program (Laskowski *et al.*, 1993). The numbering refers to the secondary structure element. Motif 1 is coloured in light yellow, motif 2 is in green and motif 3 is in orange.

structures of AspRS, that of pkAspRS contains (i) an N-terminal domain corresponding to a standard OB-fold, formed by a five-stranded β -barrel with an α -helix between strands S3 and S4; (ii) a hinge domain, residues 100–131; and (iii) the active site domain, built around a seven-stranded β -sheet, characteristic of class II aminoacyl-tRNA synthetases (Figure 1). As illustrated by sequence alignments based on the present three-dimensional structure analysis, pkAspRS shows characteristics of both the bacterial and eukaryotic enzymes (Figure 2) (Imanaka *et al.*, 1995). The N-terminal domain of pkAspRS is closer to that of the bacterial enzymes than to that of the eukaryotic AspRS, with the absence of an N-terminal extension. In contrast, the catalytic domain closely resembles that of the eukaryotic enzymes. In particular, the insertion domain located between motifs 2 and 3 in the eubacterial enzymes is reduced in pkAspRS, like in yeast AspRS. Moreover, the C-terminal extension, part of the dimer interface and specific to bacterial organisms (Poterszman *et al.*, 1993; Delarue *et al.*, 1994), is also absent.

The archaeal enzyme also displays some specific features. The most important is probably the structure of the loop L1 (P81–E88; Figure 1B) of the N-terminal domain, involved in the recognition of base C36 of the GUC anticodon of tRNA^{Asp} in the yeast AspRS complex. This might be related to the tRNA specificity constraints in archaeal enzymes (Curnow *et al.*, 1996) (see below).

pkAspRS monomers are functionally asymmetric in the crystal

A complete data set was collected to 1.9 Å resolution from a single crystal soaked in a solution containing ATP, MgCl₂ and aspartic acid (Table I). A difference Fourier map with modules and phases derived from the native model revealed, in one monomer (Figure 3A), strong extra positive density into which an aspartyl adenylate could be fitted unambiguously. Surprisingly, in the other monomer (monomer 2), strong peaks of extra positive density could also be seen but clearly corresponded to an ATP molecule bound with magnesium ions (Figure 3). After rigid body and positional refinements, using the model of the apo-enzyme, an aspartyl adenylate molecule was introduced into monomer 1 and an ATP molecule with one magnesium ion corresponding to a peak at the 10 σ level in the $F_o - F_c$ map were introduced into monomer 2. Two other magnesium ions complexed to the ATP molecule were also identified, but as peaks of lower intensity, and, in order to avoid any bias, were not introduced before the very last steps of refinement (Table I) (Figure 3).

In monomer 1, the aspartyl adenylate lies in a cleft surrounded by motifs 2 and 3 and by strand A5. The adenine base of ATP is held by two H-bonds involving N6 and a π interaction with the invariant Arg412 of motif 3 and stacked by an alanine residue (Ala227, Figure 2), generally a Phe in all other class II aminoacyl-tRNA synthetases. Despite this difference, the conformation of the region surrounding Ala227 is very similar to the corresponding one in yeast AspRS (Cavarelli *et al.*, 1994). The absence of phenylalanine at this position was also reported in the case of HisRS from hamster (Delarue and Moras, 1993). The ribose moiety adopts a C3' endo conformation and is held by H-bonds between its

Fig. 2. Sequence alignment of aspartyl-tRNA synthetases from *T.thermophilus* (tth), *P.kodakaraensis* (pyc) and *S.cerevisiae* (ysc). The pro and euc lines display the consensus sequences for prokaryotic and eukaryotic enzymes respectively. The residues conserved in all AspRS sequences are in bold black. Residues conserved only in prokaryotic organisms are in bold green, and those conserved only in eukaryotic organisms are in bold red. For the consensus lines, the conservative replacements are indicated: h, hydrophobic; +, basic; -, acidic; φ, aromatic; λ, small; and ., any residue. Three gaps exist, one in the eukaryotic family corresponds to position 24 of pycAspRS, and two are specific for prokaryotes (position 171 and 218 of pyc).

3' hydroxyl group and the side chain of Glu361 on one hand, and its 2' hydroxyl group and the main chain carbonyl of Ile362 on the other. The side chain of the class II invariant Arg214 bridges the carbonyl group of aspartate and the α-phosphate. The specificity towards the aspartic acid side chain is achieved mainly by Arg368 and Lys195, making hydrogen bonds with the carboxylate group. These in turn are stabilized by interactions with Glu233 and Asp231 of motif 2. These four residues are strictly conserved in all AspRSs. As shown in the yeast system, when Asp is replaced by Asn, Arg368 and Lys195 each contribute tens of kcal/mol to the binding free energy difference, favouring Asp. Changing just Lys is thought to be energetically sufficient to bring the affinity of Asn up into the same range as that of Asp, but is probably insufficient to make it bind in a reactive geometry (Archontis *et al.*, 1998). The amino group of aspartate is held by three hydrogen bonds, with Gln192, Glu170 and water molecule bridging NH_3^+ to the side chain of Asp231 (Figure 4). Additional solvent-mediated interactions involving Ser229 and Ser364 also contribute to the binding of aspartyl adenylate (Figure 4). Similar interactions were observed in AspRS from *T.thermophilus* complexed with aspartyl adenylate (Poterszman *et al.*, 1994) and in a SerRS–seryl adenylate complex (Belrhali *et al.*, 1995). However, in contrast to SerRS, no metallic ion is seen to interact with the α-phosphate of aspartyl adenylate in pkAspRS, as shown on the ATP-Mn²⁺-soaked crystals. In the pkAspRS–aspartyl adenylate complex, a water molecule bound to the α-phosphate group was identified (Figure 4).

In monomer 2, aspartyl adenylate is not formed, and an ATP molecule complexed with three magnesium ions occupies the active site (see below). The superimposition of monomer 1 on monomer 2 leads to an r.m.s. deviation of 0.789 Å for the 438 pairs of C_α atoms compared. The most remarkable difference corresponds to the conformation of the loop located downstream from motif 1, called the 'flipping loop' (residues 167–173, Figure 1). In monomer 1, the conformation of the loop is closed. Hence, the side chain of Glu170 interacts with the NH_3^+ group of the aspartic acid moiety of adenylate. In monomer 2, the loop is also well defined but its conformation is open, therefore prohibiting an interaction with the amino acid moiety of adenylate. Analysis of the crystal packing reveals strong interactions between the 'flipping loop' of monomer 2 and a loop from the N-terminal domain of a symmetry-related molecule. As a consequence, the flipping loop of monomer 2 is locked into an open conformation. Accordingly, in the crystals of apo-enzyme, the flipping loop appears mobile in monomer 1 and ordered in monomer 2. These observations suggest that this loop is necessary to the binding of the aspartyl moiety and, therefore, to the formation of adenylate. This conclusion is confirmed by the structure of the complex with the aspartic acid substrate (see below). Note that a similar positioning of the loop was observed in the crystal structure of the enzyme from *T.thermophilus*, analysed at 4°C (Poterszman *et al.*, 1994).

ATP binding

In order to obtain further insight into the role of the flipping loop and to confirm the role of magnesium ions

Fig. 3. Fragment of the 1.9 Å resolution $2F_o - F_c$ map contoured at 1.5σ . (A) Aspartyl adenylate within the active site of monomer 1 of pkAspRS. (B) The ATP-Mg²⁺ molecule in monomer 2 of pkAspRS, the three magnesium ions are in yellow and the liganded water molecules are in red. In this drawing, the Ser364 side chain is oriented towards interaction with a phosphate group of ATP-Mg²⁺. For the sake of clarity, the alternative conformation was omitted but is clearly suggested by the electron density. (C) The aspartic acid within the active site of monomer 1 of pkAspRS. The figure was drawn using the program 'O' (Jones *et al.*, 1991).

in the conformation of the ATP molecule, we solved the structure of pkAspRS complexed to ATP and MnCl₂. Data were collected to 1.9 Å resolution from a single crystal. A continuous and highly contrasted density present in both monomers could be identified readily as an ATP molecule complexed with three manganese ions by calculating a difference Fourier map with coefficients $F_o^{ATP:Mn} - F_c^{Nat}$. This model was refined at 1.9 Å resolution (Table I). Structural analysis of this complex clearly

shows that ATP-Mn²⁺ molecules are in exactly the same conformation in both monomers. Moreover, the conformation of the bound ATP-Mn²⁺ molecule is also identical to the conformation of the bound ATP-Mg²⁺ molecule. Enzyme residues are in similar positions, and the three manganese sites are superimposable on the magnesium ones. The structures of the enzyme complexed with either ATP-Mn²⁺ or ATP-Mg²⁺ are fully superimposable (r.m.s. deviation 0.2 Å).

Fig. 4. Stereo views of the active site-bound substrates: (a) aspartic acid (orange), (b) ATP (blue) and (c) aspartyl adenylate (green). The residues involved in the substrate positioning and the corresponding hydrogen bond interactions (dotted lines) are shown. Water molecules are in yellow, magnesium atoms in magenta. Residues with names in violet are strictly conserved amongst all AspRS sequences (Program Setor; Evans, 1993).

The ATP molecule adopts the class II-specific U-shaped conformation, with the pyrophosphate moiety of the molecule bent towards the adenine ring (Cavarelli *et al.*, 1994; Belrhali *et al.*, 1995; Arnez *et al.*, 1997). The γ -phosphoryl is held by three H-bonds involving the two NH groups of the invariant Arg412 of motif 3, and N ϵ of His223 of motif 2. A weaker interaction with the highly mobile Arg222 is also observed. Arg222 and His223 are both conserved in all eukaryotic AspRSs sequences. The α -phosphoryl forms H-bonds with the two NH groups of the invariant Arg214 of motif 2. In addition, the bent conformation is stabilized by three hexacoordinated magnesium ions (Figures 3 and 4). In contrast to the case of the SerRS-ATP-Mn²⁺ complex (Belrhali *et al.*, 1995), the strongest magnesium or manganese site (Mg1) is that bound to the β - and γ -phosphates (Table I). Four water molecules complete the coordination sphere of Mg1, one of them also bridges N7 of the adenine ring, thereby stabilizing the bent conformation of ATP. The

side chains of Glu216 and Glu174 also contribute to the stability of the hydrated magnesium. Mg2 bridges the α - and β -phosphoryl oxygens, two water molecules and the side chains of Glu361 and Ser364 completing the hexacoordination. Interestingly, an alternative conformation of Ser364 which could explain the high mobility of this magnesium ion is clearly visible in the electron density map (Figure 3). Ser364 can interact directly either with the magnesium ion (2 Å) or with the α -phosphate of ATP (2.6 Å). Such alternative conformations are probably of functional importance during the catalysis, where interaction with the α -phosphate group could be reinforced, thus favouring the stability of the transition state. The octahedral coordination of Mg3 is achieved by three water molecules, β - and γ -phosphoryl oxygens and Glu361 (Figure 4). Note that Mg2 and Mg3 share one phosphoryl oxygen (β), one water molecule and the side chain of Glu361 (Figure 4).

Fig. 5. The movements of the flipping loop and the motif 2 loop in the different states of the enzyme. (a) Apo-enzyme alone (red), and superimposed (C_{α} of the catalytic domain) on (b) the structure of the pkAspRS–aspartic acid complex (blue), (c) the structure of the pkAspRS–ATP- Mg^{2+} complex (green) and (d) the pkAspRS–aspartyl adenylyate complex (yellow). Movements of the side chains and of the mobile loops are indicated by a black arrow.

Aspartic acid-binding site

A complete data set to 1.95 Å resolution was collected from a single crystal soaked in the presence of aspartic acid. By using the native structure as the starting model, unambiguous continuous density corresponding to an aspartic acid molecule was present in $F_o - F_c$ and $2F_o - F_c$ maps in monomer 1 but not in monomer 2. This was confirmed by further refinement in the absence of added substrate. Thus, the amino acid substrate was finally introduced into monomer 1 during the last steps of refinement, while water molecules were positioned to account for the extra positive peaks identified in the second monomer (Figure 3; Table I).

In monomer 1, all interactions involved in the recognition of the aspartic acid side chain, as identified in the pkAspRS–aspartyl adenylyate structure, are present (Figure 4). Moreover, the binding of the aspartic acid substrate is accompanied by two major conformational changes. First, the flipping loop, as described in the case of the aspartyl adenylyate complexed structure, adopts a closed conformation with the side chain of Glu170 bound to the amino group of aspartic acid. Secondly, the amino acid carboxylate group is bound to the side chain of Ser364 and one NH group of Arg214 of motif 2, which moves away from its position in the apo-enzyme. The second NH group of Arg214 remains free and ready to interact with the ATP substrate. The network of water molecules described in the structure of the complex with aspartyl adenylyate is also well conserved (Figure 4).

In monomer 2, where no aspartic acid is found in the active site, the flipping loop remains in the locked opened conformation, and the side chain of Arg214 is in a

conformation identical to that encountered in the apo-enzyme. Therefore, the contribution of the flipping loop is essential to the binding of the aspartic acid and the subsequent formation of aspartyl adenylyate.

Discussion

Conformational changes associated with the binding of ATP and aspartic acid

Analysis of the four different states of the aspartate activation reaction allows a description of the molecular mechanism at the atomic level (Figure 6). ATP and aspartic acid can bind separately, the role of the flipping loop and of motif 2 loop residues being crucial for amino acid binding (Figure 5). Indeed, locking of the flipping loop into an open conformation, as observed in the second monomer of the pkAspRS enzyme, is sufficient to prevent a significant binding of the amino acid substrate and abolish the catalysis.

When compared with the structure of the apo-enzyme, the substrate-bound proteins exhibit significant but localized conformational changes (Table II), which brings the side chains of Glu216, His223 and Arg222 into contact with ATP (Figure 5). Many additional movements of side chains are observed. Two invariant arginine residues, Arg214 (motif 2) and Arg412 (motif 3), which occupies the ribose-binding site of the free enzyme structure, swing from their original position to bind the α - and γ -phosphates (Figure 5). Arg412 plays a dual role by stabilizing the adenine ring through a π interaction. Some other residues such as Asp354, Glu361 and Glu174 are also displaced to form direct water-mediated interactions with the metal

Fig. 6. Relative position of the substrates. The structures of pkAspRS complexed with ATP-Mg²⁺, aspartic acid and aspartyl adenylate, respectively, were superimposed by fitting the C_α atoms of the catalytic domain. (A) Relative location of aspartic acid and ATP-Mg²⁺ resulting from the superimposition. (B) Stick representation of the aspartyl adenylate molecule. (C) Superimposition of the three substrates. (D) Putative transition state leading to the adenylate formation according to the superimposition described above. The side chains of key residues as well as the putative trigonal bipyramid of the pentacovalent intermediate (dashed blue lines) are shown.

ions. Interestingly, the motions of the side chain of Glu174, at the C-terminal part of the flipping loop, and of Glu216 (motif 2 loop) are concerted (Figures 4, 5 and 6). It appears, therefore, that ATP is bound through an induced fit mechanism. The identical conformation of the ATP molecules in both monomers allows us to exclude that

the lack of aspartyl adenylate formation in monomer 2 was due to a non-productive binding of ATP. Moreover, in the presence of the ATP molecule only, the flipping loop in the first monomer is in an open conformation, with high *B*-values similar to those in the apo-enzyme. This rules out a significant role for the flipping loop in productive binding of the ATP molecule.

The binding of aspartic acid can be divided into two parts. The side chain is anchored to a pre-defined pocket and does not require any significant motion of enzyme residues. In contrast, binding of the peptidyl part requires some adaptation of the protein. The main conformational changes involves the flipping loop (residues 167–172) which moves from an open to a closed state to secure the aspartic acid into an active conformation. This stabilization occurs through an interaction between the NH₃⁺ group of aspartic acid and the carboxylate group of Glu170. A concomitant movement of the side chain of Arg214 allows an interaction with the main chain carboxylate group of the aspartic acid. The catalytic role of the flipping loop appears to be conserved in all AspRSs (Cavarelli *et al.*, 1994; Poterszman *et al.*, 1994). Moreover, in LysRS, the different positions observed for the corresponding loop in various complexes (Onesti *et al.*, 1995; Cusack *et al.*, 1996) suggest an identical role.

Stabilization of the transition state

A striking observation revealed by this study is that bound aspartic acid and ATP are fully superimposable on their corresponding moieties in the aspartyl adenylate complex (Figure 6). This means that before the catalytic step, the reactive oxygen of the carboxylate group of aspartic acid is located at 2.4 Å from the α-phosphate of ATP. Such an arrangement leads to a reaction occurring through an in-line mechanism with inversion of configuration at the α-phosphorus. The pentacovalent intermediate would be a trigonal bipyramid, with the entering and leaving groups occupying apical position. As shown in Figure 6, only minor movements of substrate atoms from their observed positions are necessary to reach such a transition state. Furthermore, stabilization of the reaction intermediate also requires the decrease in the negative charge of the α-phosphate. The positive charges carried by the guanidinium groups of Arg222, Arg214 and Arg412, the imidazole ring of His223 and the magnesium ions altogether participate in the delocalization of the negative charges of the triphosphate moiety of ATP.

Arg214, a strictly invariant residue among class II enzymes, and Ser364, specific of archaeal and eukaryotic AspRSs, deserve a special mention. The binding of one substrate (ATP-Mg²⁺ or Asp) orients their side chains in positions favouring their interaction with the second substrate. This feature should result in a positive coupling which might at least partly compensate for the electrostatic repulsion between the negative charges of the attacking carboxylate group and the receiving α-phosphoryl, thereby preparing the catalytic step. Such positive couplings were shown biochemically in several class I and class II aminoacyl-tRNA synthetases (Blanquet *et al.*, 1975; Holler *et al.*, 1975). The involvement of these residues in the stabilization of the transition state of the reaction is supported further by the analysis of the two possible conformations of Ser364 observed in the pkAspRS–

ATP-Mg²⁺ complex. In the first conformation, it provides the sixth ligand of Mg²⁺, whereas in the second one it points towards a phosphoryl oxygen. Interestingly, only the second conformation occurs within the amino acid and the aspartyl adenylate complexes. It is likely, therefore, that this second conformation stabilizes the transition state and facilitates the release of the pyrophosphate group with the bound magnesium ions.

Role of Mg²⁺

This structural analysis suggests a precise functional assignment for the three magnesium ions. Mg₂, which bridges α - and β -phosphates whose bond is disrupted by the reaction, is the ideal candidate for a major catalytic role. It assists the nucleophilic attack by pulling electrons away from the phosphate atoms while its anchoring to the protein would be weakened by the switch of the Ser364 side chain after binding of the amino acid carboxylate. Mg₁ and Mg₃ would also contribute to the withdrawal of electrons from the phosphate group, but their contribution to the reaction is also more structural. These ions stabilize the U-shaped conformation; Mg₁, which also binds the N7 atom of adenine, maintains the β -phosphate group in an optimal position for the on-line attack on the α -phosphate by the amino acid carboxylate group and facilitates the release of the pyrophosphate group.

Archaeal characteristics

The present study confirms that this archaeal enzyme is closer to the eukaryotic kingdom than to the bacterial one. In particular, several functionally important residues, such as Arg222, His223, Tyr339 and Ser364, are only conserved in archaeal and eukaryotic enzymes (Figure 1). As for the flipping loop, an additional glycine residue is inserted in both kingdoms. These differences are clearly of evolutionary relevance, since structural adaptations are found in the bacterial enzymes to allow side chains from other regions to play the corresponding role. For instance, in the *T.thermophilus* enzyme, the role of Ser364 is played by His442 (Poterszman *et al.*, 1994), brought into the correct position by the larger 425–466 loop (Figure 1). This provides support for the idea that the resemblance between the translation apparatus of archaea and eukarya extends to the levels of protein structure and catalytic mechanism (Dennis, 1997; Olsen and Woese, 1997).

A surprising archaeal characteristic concerns the conserved aromatic residue of motif II, predominantly a phenylalanine, on which the adenine base of ATP stacks. In all archaeal AspRSs sequenced so far, it is replaced by non-aromatic hydrophobic residues (Ala, Val, Ile), Ala227 in the case of pkAspRS. Note that this change does not affect the positioning of the adenine moiety. This peculiarity is not observed in other class II aminoacyl-tRNA synthetases.

An interesting characteristic relates to the tRNA specificity of archaeal AspRSs. Indeed, the absence of an AsnRS has been noted in some genomic sequences of archaea. A transamidation reaction providing Asn-tRNA^{Asn} from Asp-tRNA^{Asn}, evidenced in the case of the archaea *Haloflex volcanii* and providing another pathway, can compensate for the missing AsnRS and explain its absence (Curnow *et al.*, 1996). Though some archaea contain AsnRS, their genome shows the presence of

Fig. 7. (A) Binding of the GUC anticodon loop in the yeast aspartyl-tRNA synthetase complexed with yeast tRNA^{Asp}. The loop involved in the recognition of the C36 base is coloured in yellow. (B) Model for the binding of the GUC anticodon loop of the yeast tRNA^{Asp} in the *Pyrococcus* aspartyl-tRNA synthetase.

transamidases, suggesting the co-existence of two pathways to Asn-tRNA^{Asn}. In this context, it can be expected that tRNA^{Asn} is recognized and aspartylated by the archaeal AspRS. In the case of the aspartic system, the nucleotidic identity determinants governing the specific recognition of tRNA^{Asp} by AspRS are mainly the GUC anticodon and the G73 discriminator base (Putz *et al.*, 1991). Notably, all known archaeal tRNA^{Asn}, with the exception of that from *Methanobacterium thermoautotrophicum*, have a G at position 73. Therefore, the only determinant difference lies in the anticodon, and archaeal AspRS should be able to accommodate either a GUC or a GUU anticodon. In yeast AspRS, a loop specifically recognizes C36 through backbone contacts and discriminates against a U at this position (Cavarelli *et al.*, 1993). It is thus remarkable that the corresponding loop (L1 in pkAspRS) is shorter. Modelling the interaction of a tRNA anticodon on pkAspRS from the known structure of the complex in the yeast system indicates that shortening of this loop renders pkAspRS insensitive to the presence of a C or U at position 36 of its tRNA substrate (Figure 7).

Materials and methods

Expression, purification and crystallization

The gene coding for AspRS from *Pyrococcus kodakaraensis* KOD1 (pkAspRS) was cloned initially in the pET8c vector (Imanaka *et al.*, 1995; Fujiwara *et al.*, 1996). In order to improve the expression of the protein, an *Xba*I–*Hind*III fragment from this plasmid was cloned between the corresponding sites of pUC18Fatg (Schmitt *et al.*, 1996). The resulting plasmid, called pUC18AspKOD1, therefore contains the gene encoding pkAspRS under the control of both the *lac* promoter and the translation initiation signals of gene 10 of bacteriophage T7. JM101Tr cells (Hirel *et al.*, 1989) harbouring pUC18AspKOD1 were grown in LB medium in the presence of 50 µg/ml ampicillin and 0.3 mM isopropyl-β-D-thiogalactopyranoside for 24 h at 37°C. pkAspRS was purified from a 1 l culture as follows. The harvested cells were resuspended in a solution containing 10 mM Tris–HCl pH 7.5, 10 mM 2-mercaptoethanol and 20 mM KCl, and disrupted by ultrasonic disintegration. After centrifugation, the supernatant was heated for 10 min at 85°C and centrifuged again at 12 000 r.p.m. for 15 min. The supernatant was then treated with streptomycin sulfate (0.3% w/v) in order to precipitate nucleic acids. The enzyme was precipitated with ammonium sulfate (80% of saturation), redissolved in 2 ml of buffer A (10 mM Tris–HCl pH 7.5, 10 mM 2-mercaptoethanol, 100 mM KCl) and dialysed against buffer A. The solution obtained was loaded on a Superdex-200 molecular sieving column (Pharmacia; 1.6×60 cm) and eluted with buffer A at 0.4 ml/min. As expected, pkAspRS behaved as a 105 kDa dimer. The pooled fractions were loaded onto a Q-Sepharose anion exchange column (Pharmacia; 1.6×10 cm) equilibrated in buffer A. A KCl gradient was then developed from 200 to 600 mM (200 mM/h, 2.5 ml/min). pkAspRS eluted at 350 mM KCl. The enzyme obtained was homogeneous as judged by SDS–PAGE analysis. Protein concentration was deduced from the absorbance at 280 nm using a computed specific extinction coefficient of 1.09 cm²/mg. Usually, 4–6 mg of protein were obtained from a 1 l culture. The kinetic parameters of this enzyme were measured for the isotopic [³²P]PPi–ATP exchange reaction. At 25°C, in standard buffer T (20 mM Tris–HCl, 7 mM MgCl₂, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 100 mM KCl) containing 2 mM [³²P]PPi, 2 mM ATP and 2 mM aspartic acid, the isotopic exchange rate value was 3.9 ± 0.2/s. Moreover, an enzymatic assay was performed in the aminoacylation reaction by using purified *Escherichia coli* tRNA^{Asp} as substrate. At 25°C, in buffer T containing 24.5 µM [¹⁴C]Asp and 11.4 µM *E. coli* tRNA^{Asp}, the rate value was 0.010 ± 0.005/s. Although no attempt was made to optimize the assay conditions, these results were, however, consistent with the measurements reported in Imanaka *et al.* (1995). Moreover, they show that the pkAspRS enzyme is able to aminoacylate *E. coli* tRNA^{Asp}. Before crystallization assays, the enzyme was concentrated to ~5 mg/ml by using Centricon-30 concentrators (Amicon).

Crystallization trials were conducted using the hanging-drop technique at 24°C (Mac Pherson, 1982). An initial search for crystallization conditions was undertaken by using Sparse Matrix sampling (Jancarik & Kim, 1991). Crystals were obtained within 5 days in Crystal Screen I or II (Hampton Research) solutions containing either polyethylene glycol or ethylene glycol. The quality of the diffraction was checked for the two different crystalline forms. The best pattern was obtained with crystals grown in the presence of ethylene glycol. At this stage, crystallization conditions were optimized in order to improve the size of the crystals, and obtain growth at an ethylene glycol concentration ensuring effective protection against ice formation during cryocooling. Such optimized crystallization conditions were defined as 32–38% ethylene glycol in the presence of 4 mg/ml AspRS in buffer A. Under these conditions, only one or two crystals were observed in each drop within 1 week. The crystals were rod shaped with a square section, and reached 1 mm in length and 0.3×0.3 mm² in section. Analysis by polyacrylamide gel electrophoresis of the protein from washed and dissolved crystals indicated that pkAspRS was intact.

Crystal preparation and data collection

Crystals obtained using these conditions could be flash cooled directly into liquid ethane or into a nitrogen gas flow (120 K) since the concentration of ethylene glycol was sufficient to impair ice formation. Analysis of diffraction images showed that the crystals were orthorhombic, belonging to space group P2₁2₁2 with unit cell dimensions $a = 124.1$, $b = 125.1$, $c = 87.3$ Å. Four data sets were collected (Table I). The first one corresponds to an apo-enzyme crystal, the second to a soaked for 30 min at 25°C in a solution containing 35% ethylene glycol, 10 mM ATP, 10 mM MgCl₂ and 10 mM aspartic acid, the third

to a crystal soaked for 15 min in a solution containing 35% ethylene glycol, 10 mM ATP and 10 mM MnCl₂, and the fourth to a soaked for 15 min in a solution containing 35% ethylene glycol and 20 mM aspartic acid. Data were collected at –150°C by using synchrotron sources either at the ESRF ($\lambda = 1.2$ Å, D2AM beamline, Grenoble, France) with a CCD detector or at the LURE ($\lambda = 0.97$ Å, DW32 beamline, Orsay, France) on a MAR-Research phosphorimage plate system (Hamburg, Germany). Diffraction images were analysed either with the XDS program (Kabsch, 1988) or with the MOSFLM program (A.G.W. Leslie, Laboratory of Molecular Biology, Daresbury, UK) and the data processed further using programs from the Collaborative Computational Project No. 4 (1994).

Structure determination of the apo-enzyme and refinement

By using AMoRe (Navaza, 1994), two solutions could be obtained with a correlation coefficient of 17.5 and an *R*-value of 48.9%, using data in the 12–3.5 Å resolution range. These two solutions were related by a 2-fold axis corresponding to the dimer axis, thereby indicating that the asymmetric unit comprises one dimer (solvent content of 41%). From this solution, a map was calculated at 3.5 Å resolution and allowed the positioning of a polyaniline domain derived from the N-terminal domain of the *E. coli* enzyme (S. Eiler, personal communication). The N- and C-terminal domains were linked to form the initial model of the pkAspRS monomer. This polyaniline model was then improved by rigid body refinement. The crystallographic *R*-factor of this starting polyaniline model was 45.3%. The model of the apo-enzyme was refined against the 15–2.4 Å data using the program X-PLOR (Brünger *et al.*, 1987; Brünger, 1992a). A random sample containing 6% of the total data was excluded from the refinement, and the agreement between the calculated and observed structure factors corresponding to these reflections (R_{free}) was used to monitor the course of the refinement procedure (Brünger, 1992b), during which the correct side chains were added progressively. First, a round of positional refinement and simulated annealing was performed while enforcing strict non-crystallographic 2-fold symmetry. Then, stages of torsion angles refinement from 8 to 2.4 Å of resolution by using NCS restraints lowered the *R*-factor to 0.341 and the R_{free} to 0.390. Further refinement using the NCS restraints did not improve the R_{free} value. Therefore, refinement of atomic positions and of temperature factors was carried out independently for the two monomers. The refinement statistics are given in Table I. The resulting *R*-factor for the apo-enzyme structure was 21.1% and the R_{free} was 26.4%. The model shows good stereochemistry and geometry, as analysed using the program PROCHECK (Laskowski *et al.*, 1993) (Table I). All residues except Asp204 have ϕ and ψ angles within the allowed regions of the Ramachandran plot, with 91.4% in the most favoured region.

Refinement of the complexed structures

For the data sets collected either from a crystal soaked with ATP and manganese, from a crystal soaked in a solution containing ATP, aspartic acid and magnesium or from a crystal soaked in a solution containing aspartic acid, the refinement procedure was as follows. The 2.4 Å refined model of pkAspRS was used as a starting model. This model was first subjected to rigid body refinement using X-PLOR to account for slight changes in cell parameters. Then, rounds of positional refinement were performed from 15 to 2.6 Å resolution. Substrate models were built into the difference density, and other modifications of the model were made manually with the program O (Jones *et al.*, 1991). During the refinement procedure, the resolution was increased gradually to 1.9 Å. Parameter and topology files were from Engh and Huber (1991). However, additional topology and parameter files were established for ATP, aspartic acid and aspartyl adenylate. At each stage of the refinement, the model was adjusted manually. Water molecules finally were added at positive peaks over 4 σ in the $F_o - F_c$ maps, provided that they made sensible hydrogen bonds with protein or solvent atoms. A total of 1230, 965 and 408 molecules were placed in the pkAspRS–ATP–Mn²⁺, pkAspRS–Asp–AMP and pkAspRS–aspartic acid models, respectively. Results of the refinement are summarized in Table I.

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