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Yeast aspartyl-tRNA synthetase residues interacting with tRNA^{Asp} identity bases connectively contribute to tRNA^{Asp} binding in the ground and transition-state complex and discriminate against non-cognate tRNAs

Running title: analysis of yeast tRNA^{Asp}-AspRS interactions

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Abbreviations:

ARS, aminoacyl-tRNA synthetase; APS, AspRS gene; 5-FOA, 5-fluoroorotic acid

SUMMARY

Crystallographic studies of the aspartyl-tRNA synthetase-tRNA^{Asp} complex from yeast identified on the enzyme a number of residues potentially able to interact with tRNA^{Asp}. Alanine replacement of these residues (thought to disrupt the interactions) was used in the present study to evaluate their importance in tRNA^{Asp} recognition and acylation. Results showed that contacts with the acceptor A of tRNA^{Asp} by amino acids interacting through their side chain only occur in the acylation transition state, whereas those with the nearly located G73 discriminator base also occur during initial binding of tRNA^{Asp}. Interactions with the anticodon bases provide the largest free energy contribution to stability of the enzyme-tRNA complex in its ground state. These contacts also favour catalysis, by acting connectively with each other and with those of G73, as shown by multiple mutant analysis. This implies structural communication transmitting the anticodon recognition signal to the distally located acylation site. This signal might be conveyed via tRNA^{Asp} as suggested by the observed conformational change of this molecule upon interaction with AspRS. From binding free energy values corresponding to the different AspRS-tRNA^{Asp} interaction domains, it might be concluded that upon complex formation, the anticodon interacts first. Finally, acylation efficiencies of AspRS mutants in the presence of pure tRNA^{Asp} and non-fractionated tRNAs indicate that residues involved in the binding of identity bases also discriminate against non-cognate tRNAs.

Key words: AspRS, tRNA^{Asp}, specific recognition, mutagenesis, kinetic analysis.

INTRODUCTION

tRNAs and aminoacyl-tRNA synthetases play a central role in the decoding of the genetic message. These molecules mediate the accurate translation of a sequence of base triplets in an amino acid chain by highly specific codon anticodon pairing and accurate tRNA aminoacylation (Schimmel, 1987). *E. coli* contains 67 different tRNAs corresponding to the twenty amino acids. The specific recognition of each ensemble of isoacceptor tRNA by their cognate ARS is dependent on a small set of nucleotides called the identity elements (Schulman, 1991). They comprise positively interacting elements (determinants) which frequently, but not exclusively, are located in single stranded regions of the tRNA. Determinants often include the anticodon triplet and the discriminator base (position 73); in some cases, determinants also consist of peculiar phosphate-sugar backbone sequences (McClain *et al.* 1994). Moreover, there are negative elements (antideterminants) that prevents a tRNA from being recognised by a non-cognate ARS. In a few cases, repulsive or attractive effects in tRNA recognition are also dependent on base modifications (Muramatsu *et al.*, 1988; Sylvers *et al.*, 1993; Perret *et al.*, 1990; Pütz *et al.*, 1994). Negatively and positively interacting residues together with tertiary features (Hou *et al.*, 1993; McClain, 1993; Asahara *et al.*, 1994) which maintain these elements in a particular spatial presentation, collectively determine specificity, i.e. the ability of a given tRNA to be preferentially aminoacylated by a given ARS. Kinetic differences rather than binding differences control specificity of aminoacylation (Ebel *et al.*, 1973). Indeed, the free energy required for the higher acylation rate differentiating a cognate tRNA-ARS system from a non-specific one, in general is mainly provided by interactions in the acylation transition state rather than by contacts involved in initial binding of tRNA to ARS. This is well illustrated by the observation that, upon mutation of the recognition elements, the catalytic acylation rate may be changed by more than five orders of magnitude (Schulman and Pelka, 1988), whereas K_m values generally remained within a 100-fold increase.

To date, information related to tRNA-ARS recognition were mostly obtained from studies on tRNA identity elements. In the case of the aspartic system, AspRS was proposed to interact with positions 34, 35, 36 and 38 in the anticodon loop, and 73 in the acceptor arm, and at the level of the base pair G10-U25 in the stem of the D arm (Pütz *et al.*, 1991; Cavarelli *et al.*, 1993). These identity elements have been localised and characterised mostly by the effect mutations at these positions had on the acylation efficiency of a T7 tRNA^{Asp} transcript. However, it might be stressed that kinetic parameter changes observed upon base replacement and the related free energy variations may not necessarily reflect the real importance, or even the participation of the tested positions in tRNA recognition. Indeed, replacement of a given base involved or not in contacts, not only may result in disruption of the existing contacts with the wild type base, but also may lead to positive or negative interactions with the new base.

The approach used in this work to evaluate the functional importance of the tRNA^{Asp}-AspRS interactions consisted of analysing the effects of alanine replacement of the amino acids involved in these bonds on kinetic properties of AspRS. Substitution by this short side chain non-polar amino acid may reasonably be thought to abolish interactions. This paper examines all the tRNA^{Asp}-AspRS interactions identified by crystallographic studies of the tRNA^{Asp}-AspRS complex (Ruff *et al.*, 1991; Cavarelli *et al.*, 1993), with particular interest being devoted to base specific contacts. Further on, tRNA^{Asp} binding residues of AspRS were tested for their possible role in discrimination against non-cognate tRNAs. An estimate of their antideterminant capacity was proposed to be reflected by the difference between the mutation-induced k_{cat} variations measured in the presence of pure and unfractionated tRNA^{Asp}. Lastly, binding energies

corresponding to the different AspRS-tRNA^{Asp} contacts, indicate how the specific AspRS-tRNA^{Asp} complex formation may occur.

RESULTS AND DISCUSSION

The crystallographic structure of AspRS complexed to tRNA^{Asp} has been solved to high resolution (Ruff *et al.*, 1991; Cavarelli *et al.*, 1993). The enzyme contacts the tRNA along the inner part of its L-shaped tertiary structure, but only limited portions of the tRNA are in direct contact with the protein. The two main domains of the tRNA^{Asp} which interacts with AspRS are the major groove side of the acceptor stem (including the discriminator base G73 and the terminal A) and the anticodon loop. Enzyme residues interacting with the major groove of the tRNA acceptor arm are mainly located in the central loop of the conserved motif two of class II ARSs (residues 325 to 336 for AspRS). This loop makes close contacts with the discriminator base G73 and the first base pair U:A. As for the anticodon region, it is tightly bound to the terminal region of the enzyme, a domain built around a five-stranded β -barrel fold which is common to the subgroup comprising aspartyl-, asparaginyl- and lysyl-tRNA synthetases (Ruff *et al.*, 1991; Onesti *et al.* 1995; Cusack *et al.*, 1996a). In addition, contacts involving the ribose-phosphate backbone of U11 and U12 are observed with the small hinge region connecting the N-terminal to the C-terminal regions.

All the tRNA^{Asp} binding residues were individually mutated by alanine substitution, and the corresponding proteins overexpressed, purified and tested for their catalytic properties. Furthermore, double and triple mutants were constructed with alanine substitutions at residues interacting specifically with tRNA^{Asp} bases. The variation of the acylation activation energy ($\Delta\Delta G$) induced by mutation was calculated according to Carter *et al.* (1984) from the variation of the kinetic specific constant k_{cat}/K_m . The $\Delta\Delta G$ was differentiated into its two energy components $\Delta\Delta G_b$ ($\Delta\Delta G_b = -RT \ln K_d(\text{mut})/K_d(\text{nat})$) and $\Delta\Delta G_{kcat}$ ($\Delta\Delta G_{kcat} = \Delta\Delta G - \Delta\Delta G_b$): they correspond to the variations of the free energy of AspRS-tRNA^{Asp} binding in the ground state ($\Delta\Delta G_b$) and in the transition state ($\Delta\Delta G_{kcat}$). In the case of a multiple mutant, the measured free energy variations ($\Delta\Delta G$ experimental or exp.) were compared to the sum of the free energy variations obtained upon individually mutating its residues ($\Delta\Delta G$ theoretical or th.). This was to assess whether interactions with the tested residues would act independently or in an interactive manner.

Interactions with the CCA

The CCA end of tRNA^{Asp} with its 3'OH of the terminal ribose is maintained on AspRS in a position allowing attack of the α -phosphorus of the aspartyl-adenylate intermediate located in its adjacent binding pocket (Cavarelli *et al.*, 1994). Among the six residues shown to be in close vicinity of the CCA sequence of tRNA^{Asp} and interacting by their side chains, Ser280, Gln300, Ser301 Phe304 and Glu327 are thought to interact with A76, and His334 with C74 (Table 1, Fig. 1). In addition Glu 327 and His 334 interact with ATP and respectively with the N6 of the adenine ring and the α -phosphate. Finally it has to be mentioned that the terminal A also interacts with the backbone of Glu281 and Gly283.

AspRS contacts the 3' terminal A of tRNA^{Asp} mainly in the transition state

Substitution by alanine of the five residues interacting with their side chain with the 3' terminal A decreased the acylation rate constant but had no significant effect on tRNA binding (Table 2).

Thus, these residues only establish functional enzyme interactions with A76 in the transition state and do not contribute significantly to tRNA binding in the ground state. However initial binding may not be excluded: it may occur via interactions with the backbone of the Glu281 and Gly283 residues.

The C74 binding residue His334 is involved in the activation and acylation steps of the aminoacylation reaction

His334 contributes to tRNA^{Asp} binding in the ground state and favours acylation efficiency as evidenced by kinetic changes upon alanine mutation (Table 2. Substitution by alanine of this position also resulted in significant changes of the AspRS affinities for ATP and aspartic acid and impaired, more or less severely, the catalytic efficiency of the enzyme in the ATP-Pi exchange reaction (see also Cavarelli *et al.*, 1994, for other mutation types of His334). Thus, residue 334 is not just involved in the acylation step, but also contributes to the stability of the transition state of the amino acid activation reaction and participates to ATP and aspartic acid binding. The importance of this residue in catalysis may be reminiscent of the fact that it is one of the most conserved residues in the structural motif 2 of class II ARS.

Interactions with the discriminator base G73 and the acceptor arm

The ability of a tRNA to establish productive contacts with an ARS in the transition state is dependent on the relative positions of these molecules in the ground state complex. This positioning depends on specific contacts of the enzyme with the so called determinant or identity elements of the cognate tRNA. The latter commonly are found in the anticodon loop and in the acceptor stem, including the discriminator base at position 73. The discriminator, the only variable residue found near the site of aminoacylation, was shown to contribute to the identity of virtually every tRNA species (McClain, 1993a). It acts either as a positive recognition element towards the cognate enzyme and/or by promoting negative interactions against non-cognate ARS. Co-crystal structures of tRNA-aaRSs complexes showed that positive recognition between the discriminator base and the enzyme may occur via direct interactions (as for the Asp system) and/or indirectly by influencing the positioning of the acceptor end without interacting with the enzyme (Hou, 1997).

AspRS interactions with G73 act cooperatively, with coupling energy in favour of the acylation transition state

Protection assays by AspRS against chemical modifications and mutational analysis of tRNA^{Asp} transcripts indicated that G73 is a major identity element of tRNA^{Asp} (Romby *et al.*, 1985; Pütz *et al.*, 1991; Rudinger *et al.*, 1992). Crystallographic studies of the AspRS-tRNA^{Asp} complex showed that chemical groups of the discriminator base are in direct interaction with three residues of the enzyme (Asn328, Ser329 and Thr331) (Table 1, Fig. 1), (Cavarelli *et al.*, 1993). Due to their position in the variable loop of motif two (Eriani *et al.*, 1990a), they are strongly suspected to contribute to tRNA recognition. Indeed, the important variability of this domain in amino acid number and composition through different ARS (Ruff *et al.*, 1991) clearly points to its idiosyncratic role in selectivity of tRNA binding.

Residues in the acceptor stem, and particularly at position 73, have been clearly shown to strongly influence the efficiency of the acylation reaction (Hou, 1997). In the case of AspRS, as reported here, single mutations N328A, S329A or T331A mainly affected tRNA binding in the ground state (from 3 to 7-fold) and only moderately changed the acylation *k_{cat}* of the enzyme. By summing the free energy variations measured for each single mutant, it appears that the three interactions collectively contribute by 2.54 kcal/mol to tRNA binding in the ground state

($\Delta\Delta G_b$) and only by 0.47 kcal/mol to the acylation efficiency ($\Delta\Delta G_{kcat}$). However, the corresponding AspRS triple mutant having substitutions at the three previous positions, displayed free energy variations mainly in the transition state ($\Delta\Delta G_{kcat} = 2.49$ kcal/mol) and to a lesser extent in the ground state of the enzyme-tRNA complex ($\Delta\Delta G_b = 1.81$ kcal/mol) (Table 3 and Fig. 2); the overall free energy variation ($\Delta\Delta G_{exp.} = 4.3$ kcal/mol) is higher than the one obtained by adding free energy values from single mutants ($\Delta\Delta G_{th.} = 3.01$ kcal/mol). These results show that the three residues do not interact independently with G73 but in a connective manner. The coupling effect favours the acylation rate ($D\Delta\Delta G_{kcat} = 2.02$ kcal/mol) to the detriment of the tRNA^{Asp} binding strength in the ground state ($D\Delta\Delta G_b = -0.73$ kcal/mol). Thus, and as has been generally observed for other synthetases, the contribution of position 73 to tRNA^{Asp} aminoacylation specificity is also mainly realised in the acylation transition state. Connectivity implies formation of new contacts associated with structural rearrangements, which, in the present case, result in a better functional orientation of the acceptor end towards the active site. The crucial influence G73 has on the reactivity of the terminal A relies on the important role it plays on the stability of the acceptor arm. Previous studies showed that the discriminator base modulates the interaction strain between the first base pair of the tRNA and also stabilises the single strand acceptor end by a stacking effect, thus determining its conformation (Limmer *et al.*, 1993; Puglisi *et al.*, 1994).

Contacts with ribose and phosphate groups in the acceptor arm contribute to tRNA binding in the ground state

Nucleotide 73 also interacts with residues Ser423 and Thr424, both being located in a loop of the variable region connecting motifs 2 and 3. These contacts may be qualified as "non-specific" with respect to the tRNA since they involve the phosphate backbone. Their suppression had only little or no effect on the enzyme acylation *kcat*. A similar result was obtained upon mutation of the A72 phosphate binding residue Lys428.

Another protein-tRNA contact involves the phosphate group adjacent to U1 of the tRNA, located on one monomer of the enzyme, and Lys293 of the other subunit, a cross interaction which is peculiar to the aspartic system. Changing Lys293 to alanine affected the affinity for tRNA^{Asp} (11-fold decrease), and only had a limited effect on the acylation efficiency (3-4 fold increase) despite being well conserved in the AspRS sequences (Eriani *et al.*, 1990b).

The last interaction with the acceptor arm involves the phosphate of C67 and Lys553. Mutation of this lysine decreased the affinity of AspRS for the tRNA^{Asp} by ten-fold but had no effect on the acylation *kcat*.

Interactions with the central region of the tRNA

Five interactions can be observed with the central core of the tRNA (Table 1, Fig. 1). They involve residues located in the N-terminal part of the β -barrel-like domain of the enzyme (Lys155 and Glu202) and in the hinge region (Asn227, Thr230, Asp210) which connects this domain to the C-terminal domain containing the active site. All these interactions are not specific of the tRNA^{Asp} bases, and individual substitutions by alanine residues lead only to effects on tRNA^{Asp} binding (*K_d* increased by 4 to 10-fold). These contacts, as suggested by crystallographic data, maintain tRNA^{Asp} on the AspRS molecule in a configuration favouring optimal presentation of the identity elements for interaction with their counterpart residues on the enzyme. It may be noticed that no interactions could be detected with the base pair G10:U25 (Cavarelli *et al.*, 1993) suggesting that the effects on acylation observed upon mutation of these positions in the tRNA^{Asp} transcript (Pütz *et al.*, 1991) probably are due to alteration of

the three-dimensional structure of the tRNA, triggered by changes at the level of the G10:U25:G45 tertiary interactions.

Interactions with the anticodon loop

Nucleotide transplantation assays into host tRNAs revealed that tRNA^{Asp} contains four major recognition elements in its anticodon loop: the anticodon triplet (G34, U35, C36) and base C38 (Pütz et al., 1991; Frugier *et al.*, 1994). These bases, as evidenced by crystallographic studies, are interacting with eight residues located on the different strands of the β barrel domain. They involve the highly conserved residues Arg119, Phe127, Gln138 and Glu188. These latter were shown to be also conserved in asparaginyl- and lysyl-tRNA synthetases, suggesting that they may constitute the basic module for recognition of the anticodon motif XUX (Eriani *et al.*, 1990b). Two additional interactions involve the adjacent bases U33 and m1G37.

Individual mutation of anticodon-binding residues strongly affected tRNA^{Asp} binding without marked effect on acylation k_{cat}

Results in Table 2 show that alanine substitution of each of the ten residues interacting with the anticodon region of tRNA^{Asp} predominantly changed the affinity for tRNA^{Asp}, without any marked effect on the acylation k_{cat} . It may be noticed that these changes, in general, appear to be more important than the one obtained upon disruption of contacts with the discriminator base. The strongest affinity decrease (35-fold) was observed by suppression of the stacking interaction between F127 and U35 upon FA127 mutation, its free energy (2.09 kcal/mol) being higher than the one of the other anticodon contacts (0.81-1.56 kcal/mol) (Table 3). In *E. coli* LysRS, mutation of the corresponding position (Phe85) caused a marked drop in aminoacylation efficiency. It was thought to stack the U35 of tRNA^{Lys} in a similar manner to that described for either the yeast AspRS-tRNA^{Asp} or *Thermus thermophilus* LysRS-tRNA^{Lys} crystalline structures (Commans *et al.*, 1998). This ring-ring hydrophobic interaction, although not base specific, may still be reasonably assumed to be involved, at least indirectly, in anticodon recognition, due to its central position within the anticodon interaction framework. Parallel stacking interactions between aromatic residues have also been reported in the *Thermus thermophilus* SerRS-tRNA^{Ser} complex (Cusack *et al.*, 1996). In that case, the hydrophobic contact between Phe262 and two consecutive bases in the acceptor stem appeared to be conserved whatever the isoacceptor serine tRNA present in the complex with SerRS. This strongly suggests a direct role of this hydrophobic interaction in specific tRNA recognition.

Interactions with the anticodon bases act in a connective way between themselves and with the G73-AspRS specific contacts. The coupling effect is mostly in favour of the acylation k_{cat}

Analysis of double mutants, having alanine substitutions of anticodon interacting residues, showed significant differences between the theoretical and experimental $\Delta\Delta G_{kcat}$ and $\Delta\Delta G_b$ values (Fig. 2). Thus, there is functional connectivity between the tested interaction pairs, implying that bases or amino acid residues involved in these contacts are structurally linked. This is well corroborated by previous observations showing that the anticodon contacts collectively trigger a significant conformational change of the anticodon region by unstacking of bases U33 to m1G37 and resulting in the protruding out of the anticodon bases (Cavarelli *et al.*, 1993). As shown in Fig. 2, the coupling effects of all interaction pairs favoured the acylation transition state and mostly lowered tRNA binding in the ground state.

Double mutant analysis also revealed that anticodon interactions are acting connectively with the G73 bonds (Fig. 2) with coupling energies mostly in favour of k_{cat} (0.73 to 2.62 kcal/mol).

Collectively these results revealed that nucleotide specific contacts at the anticodon level not only bind the tRNA in the ground state (as would be concluded from single mutant analysis) but also stimulate acylation catalysis. By acting in a synergistic way with themselves and with the G73 bonds, they induce local and distal structural rearrangements, optimising the reactive position of the acceptor stem. In other words, anticodon interactions upon AspRS-tRNA^{Asp} complex formation participate to structural distortions favouring the acylation transition state. The existence of structural connections transmitting anticodon recognition to the acceptor end are further supported by the following observations: 1- Previous studies on tRNA^{Asp} identity elements revealed that substitution of any anticodon nucleotide of tRNA^{Asp} severely impaired the *k_{cat}* of the acylation reaction. Thus, perturbation of the anticodon interaction framework is transmitted to the acylation site. 2- It was previously shown that AspRS no longer acylates tRNA^{Asp} having an anticodon amber triplet, but aminoacylation was recovered by substituting AspRS residues located near to the acceptor stem binding domain of AspRS (Martin, 1995). The fact that the activity could be rescued by mutations at that level is a clear indication that the changes effected on the anticodon interactions have been transmitted to the acceptor site, impairing its productive configuration.

Coupling of the anticodon recognition signal to catalysis has also been demonstrated for the *E. coli* GlnRS-tRNA^{Gln} (Sherman *et al.*, 1996; Kitabatake *et al.*, 1996) and MetRS-tRNA^{Met} systems (Gale and Schimmel, 1996). For the MetRS system, the part of the free energy provided by anticodon-binding which is used for the distortion effect, and applied to raise *k_{cat}*, has been established. It was shown that nearly all of the free energy of strain (7 kcal/mol) was utilised to reduce the apparent free energy needed for formation of the acylation transition state (Gale and Schimmel, 1996). An estimate of this energy in the case of the AspRS system awaits the determination of its binding constants for the anticodon and acceptor moieties of tRNA^{Asp}.

No data are available concerning the molecular pathway by which correct anticodon recognition is coupled to functional acceptor stem binding in the active site of AspRS. However, crystallographic data of tRNA^{Asp} in its free state and bound to AspRS might suggest that transfer of information to the active site at least may occur via the tRNA^{Asp} molecule (Westhof *et al.*, 1985, Ruff *et al.*, 1991). Indeed, when complexed with AspRS, tRNA^{Asp} exhibits a more pronounced bending of the L-shaped fold than in its free state, resulting from a torsion of the anticodon stem at the level of the base pair G30:U40 (Cavarelli *et al.*, 1993). As for the AspRS protein, the question of whether it contributes to the transmission of the anticodon recognition signal remains to be elucidated. To date, no significant conformational changes of AspRS upon tRNA^{Asp} binding could be evidenced to support this possibility.

AspRS residues interacting with tRNA^{Asp} identity bases also act as antideterminants against non-cognate tRNAs

Then using unfractionated instead of pure tRNA^{Asp} from yeast cells, much lower aminoacylation efficiencies (8 to 413 fold) were obtained for mutants having changes at residues involved in binding of G73 (Asn328, Ser329 and Thr331) and of anticodon bases (Arg119, Gln121, Phe127, Gln138, Lys142, Glu188), (Table 2). For double mutants, aminoacylation rates were not measurable. The obvious reason for these lower *k_{cat}* values is that some non-cognate tRNAs present in the bulk preparation are efficient competitors for the tRNA binding site of AspRS when the enzyme is mutated at the previous positions. These competitors have to be considered as nonproductive inhibitors since they appear not to be aminoacylated. Indeed, the aspartylation plateau's obtained with unfractionated tRNAs in the presence of the mutant proteins never exceeded the level corresponding to complete charging

of the amount of tRNA^{Asp} contained in the bulk tRNA preparation (results not shown). It should be mentioned that in the presence of native AspRS, aminoacylation is also less efficient when using unfractionated tRNA, although to a lesser extent than for the mutants. The acylation k_{cat} with pure tRNA^{Asp} is about twice the one obtained with total tRNA (Table 2). The increased ability of AspRS mutants to bind non-cognate tRNAs results both from their lower affinities for tRNA^{Asp} as well as from their higher affinities for the non-cognate tRNAs, as indicated by factor ρ (ρ corresponds to the affinity variation of AspRS, upon mutation, for non-cognate tRNAs present in the unfractionated pool; for calculation of ρ , see Materials and Methods).

The affinity increase of AspRS for non-cognate tRNAs when mutated at base specific residues implies that these latter also act as antideterminants against binding of nonspecific tRNAs. As it appears from data in Table 2, the strongest effects were observed for residues interacting with anticodon bases (Glu188, Arg119 and Gln121). Evidence for this dual function as positive and negative determinant was also recently demonstrated in the case of GlnRS from *E. coli*. Residue Arg343 was shown to make sequence specific hydrogen bonds with the central position of the anticodon and to interact negatively with non-cognate nucleotides present at that position (Sherman *et al.*, 1996). Thus, if one considers that chemical groups ensuring positive and negative interactions have to be on highly accessible positions, concentration of both functions on a single residue may appear as a way to optimise the discrimination system by minimising the number of residues required for specificity, and thereby reducing the structural complexity of the enzyme.

The AspRS variants mutated at A76 binding residues only displayed minor differences between their acylation k_{cat} when assayed in the presence of unfractionated and pure tRNA^{Asp}. This implies that Ser280, Gln300 Ser301 and Phe304 have neither antideterminant function nor, and consistent with our results, significantly bind tRNA^{Asp} in the ground state.

Lastly, it has to be noticed that several mutant enzymes displayed ρ values lower than 1, indicating that the substituted residues would be involved in contacts with non-specific tRNAs. In most cases these residues are interacting with the phosphate backbone of tRNA^{Asp}.

Effect of Mutations on cell growth

All the previously studied interactions were analysed for their importance on cell viability. This was realised by testing the genes coding for the corresponding mutant proteins for their ability to complement a yeast haploid strain deleted of the *APS* gene which is essential in yeast (Ador *et al.*, 1999). Thus, the growth capacity of the *APS* null strain will be dependent on the aspartylation efficiency of the mutated AspRS. In order to limit imbalance between tRNA and ARS concentrations within the cell, the mutated gene was placed in a centromeric plasmid and expressed from its own promoter. Table 2 shows that absence or low growth rates corresponded, in general, to low acylation rates (assayed with unfractionated tRNA). No cell viability was observed in the case of double mutants, a result well consistent with their very low aminoacylation efficiencies. However, the results did not permit determination of an absolute threshold below which cell growth is impaired. This is particularly well illustrated by the E327A mutant for which no growth is observed despite having nearly wild type activity (3.35 sec^{-1}) whereas several other mutants, with activities far below this level, can complement the disrupted yeast. This apparent discrepancy might rely on the possibility that reaction conditions and tRNA concentrations in the cell might differ from the one of the *in vitro* assay. Furthermore, and as recently shown for MetRS and GlnRS (Simos *et al.*, 1996), cofactors may be present within the cell, which upon binding on AspRS, might stimulate its activity.

CONCLUDING REMARKS

The present study shows that specific contacts of AspRS with the anticodon triplet and the discriminator base of tRNA^{Asp} constrain the complex in a manner to bring the acceptor stem in the optimal reactive position. The dynamic aspect of this process remains to be explored and among others, the question of whether the two sites contact the enzyme randomly or in an ordered manner. Data from the present study would support the second possibility, with the anticodon being bound first. This may be concluded from the fact that the global free energy corresponding to binding of the anticodon region is notably higher than the one of the discriminator base interactions or of contacts with any other parts of the tRNA^{Asp} molecule (Table 2). Indeed it reasonably may be assumed that the domain having the highest global affinity will have the highest binding probability. That specific recognition of tRNA^{Asp} by AspRS first occurs at the anticodon level may further be suggested by recent structural data on the tRNA^{Asp}-AspRS complex from *E.coli*: Two types of complexes were observed presenting identical interaction patterns, but with the acceptor end outside of the active site for one of them (Eiler, S. 1998). Thus, as binding of the acceptor end to the enzyme is not a prerequisite for contact formation with the anticodon, the latter may bind first. This conclusion may be extended to the yeast system as would suggest the fact that complexes from both origins present, if excepting the acceptor stem region, the same interaction framework (Eiler, S. 1998). After being anchored to AspRS by its anticodon, tRNA^{Asp} most likely will bind by U11 and U12 of its DHU stem to the hinge domain of the enzyme, as suggested by crystallographic data. These aspecific links might bring both molecules closer to each other in a manner to allow binding of the acceptor stem in its reactive position. This process is accompanied by structural adjustments, particularly upon G73 binding, as suggested by the existence of connectivity between the anticodon and G73 interactions.

Aminoacylation assays performed in the presence of total tRNA showed that AspRS residues interacting with identity elements also act negatively against non-cognate tRNAs, with a predominant effect observed at the anticodon level. However, this barrier may not be sufficient to prevent non-cognate tRNAs from binding on AspRS, and additional elements exclusively involved in tRNA discrimination might be required. Such residues have been identified in several ARS: In MetRS from *E. coli*, two acidic residues were found having antideterminant properties (Schmitt *et al.* 1993). In TyrRS from *E. coli*, Glu152 was shown to act by electrostatic and steric repulsion (Bedouelle *et al.* 1993) and in LysRS, it recently was shown that Glu135 repulses a cytosine base when present at position 35 of the tRNA^{Lys} (Commans *et al.*, 1998).

Lastly, anticodon and acceptor stem interactions also involve interactions with the sugar phosphate backbone of the tRNA^{Asp} in addition to base specific contacts. A dozen of them could be identified from crystallographic data of the AspRS-tRNA^{Asp} complex. Their mutation led to only moderate changes of the affinity for tRNA^{Asp} without competition effects when assayed with bulk tRNA; this confirms that they do not contribute to specific recognition, their role being obviously limited to stabilisation of the E-tRNA complex.

MATERIALS AND METHODS

Materials

α -[^{35}S]dATP and γ -[^{32}P]ATP were purchased from Amersham France (Les Ulis) and L-[^{14}C]aspartic acid was from the CEA (Commissariat à l'Energie Atomique, Saclay, France). Restriction enzymes, T4 DNA polymerase, T4 DNA ligase, unfractionated yeast tRNA and T4 polynucleotide kinase were from Boehringer-Mannheim (France SA, Meylan). tRNA^{Asp} was purified from yeast cells, a gift from Dr. G. Keith of our laboratory. T7 DNA polymerase was from Pharmacia. Oligonucleotides were synthesised on an Applied Biosystems 381A DNA synthesiser using the phosphoramidite method.

Mutagenesis and purification of the mutated AspRS proteins

Mutagenic primers were synthesised on a fully automated DNA synthesiser (Applied Biosystem 381A DNA). Mutagenesis of the AspRS gene, overproduction and purification of the corresponding proteins were performed according to previously described procedures (Eriani *et al.*, 1993).

Determination of the aminoacylation activity

The aminoacylation mixture (100 μl) contained 50 mM Tris/HCl, pH 7.5, 30 mM KCl, 0.1 mg/ml bovine serum albumin, 5 mM glutathione, 10 mM ATP, 0.1 mM L-[^{14}C]aspartic acid (25 Ci/mol), 20 mM MgCl_2 and 20 μg of pure tRNA^{Asp} or 300 μg of unfractionated tRNA. The reaction was initiated by the addition of enzyme and conducted at 37°C. At varying time intervals (usually 1 min), aliquots of 20 μl were spotted onto Whatman 3MM discs which finally were measured for radioactivity.

ATP-PPi exchange

The ATP-PPi reaction mixture contained 100 mM Hepes (sodium salt), pH 7.2, 10 mM MgCl_2 , 2 mM [^{32}P]PPi (1-2 cpm/pmol), 2 mM ATP, 10 mM aspartic acid. The reaction was started by enzyme addition and, after various incubation times at 37°C, 50 μl mix were measured for [^{32}P]ATP.

Determination of the affinity constant of AspRS for tRNA^{Asp}

The affinity constants of AspRS for tRNA^{Asp} was measured by the nitrocellulose filtration method (Yarus, M. and Berg, P., 1967) under experimental conditions reported by Eriani *et al.*, 1993. The binding mix (100 μl) was 50 mM potassium phosphate, pH 5.5, 10 mM MgCl_2 , 20 nM ^{32}P -labelled tRNA^{Asp} (67000 cpm) and contained various amounts of AspRS. After filtration, the nitrocellulose discs (0.45 μm , Millipore) were washed twice with 500 μl 50 mM potassium phosphate, pH 5.5, 50 mM MgCl_2 , dried and the radioactivity measured.

In vivo complementation assays

Complementation was tested by a shuffle method using the haploid YAL3 *Saccharomyces cerevisiae* strain (*a ura3-52 lys2-801^{am} trp1- Δ 63 his3- Δ 200 leu2- Δ 1 ade2- Δ 450 ade3- Δ 1483 Δ aps::HIS3, pAL3 (URA3, APS, ADE3).* This strain is chromosomally disrupted for APS and is rescued by the plasmid encoded AspRS (from pAL3). The vectors pEG93 (containing the mutated APS genes to test) were introduced into strain YAL3 by standard transformation procedures, then grown at 28°C on minimal medium supplemented with adenine (10 $\mu\text{g}.\text{ml}^{-1}$), with histidine (20 $\mu\text{g}.\text{ml}^{-1}$),

with lysine (20 $\mu\text{g.ml}^{-1}$) and with leucine (60 $\mu\text{g.ml}^{-1}$). Transformants were then subjected to a drop test on minimal medium containing 5-FOA (4 10^{-3}M), adenine (10 $\mu\text{g.ml}^{-1}$), histidine (20 $\mu\text{g.ml}^{-1}$), uracyl (25 $\mu\text{g.ml}^{-1}$), lysine (20 $\mu\text{g.ml}^{-1}$) and leucine (60 $\mu\text{g.ml}^{-1}$). In these growth conditions the rescuing pAL3 (*URA3*, *APS*, *ADE3*) vector can leave the strain (medium supplemented in uracyl) if the pEG93 vector encodes an active AspRS. If pEG93 encodes an inactive AspRS, pAL3 cannot leave the cell and *URA3* will metabolise the 5-FOA into 5-FUMP toxic for the cell. By this way we were able to identify the transformants which contain active *APS* genes by their ability to grow on 5-FOA medium whereas the inactive *APS* genes gave transformants unable to grow on the same medium.

Formulation of the ρ value

ρ is the ratio between the apparent inhibition constants of non-cognate tRNAs measured with respect to native and mutated AspRS. The ρ values were calculated using the equation:

$$\rho = \frac{3.5 \times 10^{-8} (k_{\text{mut.P}} - k_{\text{mut.U}})}{(k_{\text{mut.U}} - K_{\text{mut.P}})}$$

in which $k_{\text{mut.P}}$ and $k_{\text{mut.U}}$ are the acylation k_{cat} values of the mutated AspRS in the presence of pure (P) and unfractionated (U) tRNA^{Asp} , respectively. The equation of ρ was established as follows:

Let us consider the acylation rate of native AspRS (**vn**) in the presence of unfractionated tRNA^{Asp} :

$$V_n = \frac{k_n.P \times E \times S}{S + K_{m,n}.P (1 + \frac{x.S}{K_i.n})}$$

where

- $k_n.P$ is the acylation rate of native (n) AspRS in the presence of pure (P) tRNA^{Asp} .
- E is the AspRS concentration.
- S is the tRNA^{Asp} concentration in the reaction mix.
- $K_{m,n}.P$ corresponds to the Michaelis constant of native (n) AspRS for pure (P) tRNA^{Asp} .
- $x.S$ is the concentration of non-cognate tRNAs in the acylation assay
- $K_{i,n}$ is the inhibition constant with respect to native AspRS (n) for non-cognate tRNAs

If S is high (S^*) compared to $K_{m,n}.P$ (this is the case in all our tests), we have:

$$V_n = \frac{k_n.P \times E \times S^*}{S^* + K_{m,n}.P + K_{m,n}.P \times \frac{x.S^*}{K_{i,n}}}$$

$K_{m,n}.P$ may be neglected, thus

$$V_n = \frac{K_n.p \times E}{1 + K_{m,n}.P \times \frac{x}{K_{i,n}}}$$

If considering that **Vn** corresponds to $k_n.U \times E$ ($k_n.U$ being the acylation rate constant of native AspRS in the presence of unfractionated tRNA^{Asp}), we have:

$$K_{i,n} = \frac{k_n.U \times K_{m,n}.P \times x}{K_n.P - k_n.U}$$

In the same manner as above it may be shown that:

$$K_{mut} = \frac{k_{mut.U} \times K_{mmut.P} \times x}{k_{mut.P} - k_{mut.U}}$$

and thus :

$$\frac{K_{in}}{K_{mut}} = \frac{(k_{n.U} \times K_{mn.P})(k_{mut.P} - k_{mut.U})}{(k_{mut.U} \times K_{mmut.P})(k_{n.P} - k_{n.U})} = \rho$$

Considering, as measured, that $k_{n.U} = 5 \text{ sec}^{-1}$, $K_{mn.P} = 3.10^{-8} \text{ M}$ and $k_{n.P} = 9.3 \text{ sec}^{-1}$, we will have:

$$\rho = \frac{3.5 \times 10^{-8} (k_{mut.P} - k_{mut.U})}{(k_{mut.U} \times K_{mmut.P})}$$

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FIGURES AND TABLES

Figure 1 Representation of the complex between AspRS (red) and tRNA^{Asp} (green) from yeast, with the amino acids and bases able to interact.

Figure 2 Graphical representation of results from Table 3.
Columns with "th" and "exp" respectively represent $\Delta\Delta G_{th}$ and $\Delta\Delta G_{exp}$.

Table 1 Potential AspRS-tRNA^{Asp} interatomic contacts tested for their importance in the tRNA^{Asp} acylation reaction

AspRS - tRNA ^{Asp} interacting chemical groups			
AspRS		tRNA ^{Asp}	
A. Terminal A binding residues			
Ser280	O ^γ	N6	A76
Gln300	N ^ε	O2'	A76
Ser301	O ^γ	O2'	A76
Phe304	Partial stacking		A76
Glu327	O ^ε	O2'	A76
B. Acceptor arm binding residues			
Lys293	N ^ζ	OP	U1
His334	N ^ε	N4	C74
Ser329	O ^γ	N3	C74
	O ^γ	N1	G73
Glu327	O ^ε	N2	G73
Asn328	O	N2	G73
	N ^γ	OP	U1
Thr331	O ^γ	O6	G73
Ser423	O ^γ	OP	G73
Thr424	O ^γ	OP	G73
Lys428	N ^ζ	OP	A72
Lys553	N ^ζ	OP	C67
C. Anticodon loop binding residues			
Asn117	N ^δ	OP	C38
Gln121	N ^ε	O4'	U35
	O ^ε	N3	C38
Lys180	N ^ζ	OP	m ₁ G37
	N	O2	C36
Ser181	O ^γ	O2	C36
Arg119	N ^η	O2	U35
	N ^η	O4'	C36
Phe127	Stacking		U35
Gln138	O ^ε	N3	U35
Lys142	N ^ζ	N7	G34
Glu188	O ^ε	N2	G34
Thr124	O ^γ	O4	U33
D. Central core binding residues			
Lys155	N ^ζ	OP	G30
Glu202	O ^ε	O2'	G27
Asn227	N	OP	U12
Thr230	O ^γ	OP	U12
Asp210	O ^δ	O2'	U11

All pairs of atoms were separated by < 3.5 Angström, the distance assumed to be required for direct interaction. In tRNA^{Asp}, OP are phosphate oxygens and atoms with primes belong to the ribose. All other atoms belong to AspRS amino acids or tRNA^{Asp} bases. *Lys293 belongs to the other subunit of the AspRS catalytic dimer.

Table 2 Catalytic properties of mutated aspartyl-tRNA synthetases

AspRS	$k_{\text{cat}}^{\text{exch.}}$ (s ⁻¹)	K_m		$K_d(\text{nM})$ tRNA ^{Asp}	$k_{\text{cat}}^{\text{acylation}} \text{ (s}^{-1}\text{)}$			Yeast disruptant growth
		ATP (μM)	Asp (mM)		tRNA ^{Asp}	Total tRNA	ρ	
Native	27	30	2.5	30	9.3	5	1	+
<i>A. Terminal A binding mutants</i>								
S280A	34	13	17.5	30	4.3	3	0.50	+
Q300A	10	12	7.5	25	1.7	0.56	2.80	±
S301A	16	57	60	50	2.3	1.21	0.62	±
F304A	13	6	10	30	4.6	1.8	1.80	+
<i>B. Acceptor arm binding mutants</i>								
K293A	27	6	7	350	33	12	0.17	+
H334A	0.6	290	0.25	270	1.1	0.09	1.45	+
S329A	41	26	3.5	120	12	0.77	4.24	+
E327A	5	125	30	20	3.8	3.35	0.23	±
N328A	27	9	3	85	5.2	0.66	2.85	+
T331A	20	235	4	200	5.7	0.13	7.19	±
S423A	21	30	5	25	6	2.9	1.49	+
T424A	36	31	3	120	9	5.7	0.17	+
K428A	34	23	3.5	125	14	4.9	0.52	+
K553A	32	18	4.5	280	9	5.7	0.07	+
<i>C. Anticodon loop binding mutants</i>								
N117A	31	17	3	135	15.4	5.74	0.35	+
Q121A	27	30	5	275	25	0.13	24.3	+
K180A	27	20	2	400	10.4	3.6	0.16	+
S181A	30	28	3.5	180	15	7.2	0.21	+
R119A	60	28	5.5	269	23.8	0.14	21.9	+
F127A	42	20	4.5	1050	12.4	0.03	13.7	-
Q138A	28	33	2.5	337	11	0.15	7.48	+
K142A	26	32	4	129	14.6	1.5	2.36	+
E188A	28	33	3	424	8.7	0.03	23.6	±
T124A	29	21	2.5	125	8.3	5	0.18	+
<i>D. Central core binding mutants</i>								
K155A	29	24	3	110	17.7	6.9	0.42	+
E202A	34	22	3.5	165	10.6	6.7	0.12	+
N227A	31	26	3.5	120	19.8	9	0.35	+
T230A	46	11	4.5	330	20	6.8	0.20	+
D210A	40	29	4	328	16.8	7.2	0.14	+

K_m values for ATP and aspartic acid respectively were measured in the charging reaction and in the pyrophosphate exchange reaction. K_d values for tRNA^{Asp} were measured by filtration assay according to Yarus, M. and Berg, P. (1967).

Total yeast tRNA was from Boehringer (Mannheim) (batch # 12375221-66). tRNA^{Asp} was purified from yeast cells. Differences were observed when testing other batches, suggesting a certain variability in tRNA content. +, +/- respectively indicate that cell growth is appearing after 36 and 96 hours of incubation at 28°C or not (-).

* For calculation of ρ see Materials and Methods.

Table 3 Cooperativity analysis of the G73 and anticodon binding interactions

AspRS mutants	$k_{\text{cat}}^{\text{acylation}}$ (s ⁻¹)	$K_{\text{d}}^{\text{tRNA}^{\text{Asp}}}$ (nM)	$\Delta\Delta G^a$ (kcal/mol)	$\Delta\Delta G_b^b$ (kcal/mol)	$\Delta\Delta G k_{\text{cat}}^c$ (kcal/mol)	$D\Delta\Delta G^d$ (kcal/mol)	$D\Delta\Delta G_b^e$ (kcal/mol)	$D\Delta\Delta G k_{\text{cat}}^f$ (kcal/mol)
<i>A. G73 binding mutants</i>								
N328A	5.2	85	0.95	0.61	0.34			
S329A	12	120	0.66	0.81	-0.15			
T331A	5.7	200	1.40	1.12	0.28			
N328A/S329A/T331A	0.14	650	4.30	1.81	2.49	1.29	-0.73	2.02
<i>B. Anticodon binding mutants</i>								
R119A	23.8	269	0.74	1.29	-0.55			
E188A	8.7	424	1.60	1.56	0.04			
R119A/E188A	0.88	4500	4.35	2.53	1.40	2.01	0.10	1.91
Q138A	11	337	1.33	1.42	-0.097			
E188A	8.7	424	1.60	1.56	0.04			
Q138A/E188A	4.4	2200	3.00	2.53	0.47	0.07	-0.46	0.53
K142A	14.6	129	0.59	0.86	-0.27			
E188A	8.7	424	1.60	1.56	0.04			
K142A/E188A	2	4700	4.14	3.25	0.89	1.95	0.83	1.12
F127A	12.4	1050	1.93	2.09	-0.16			
E188A	8.7	424	1.60	1.56	0.04			
F127A/E188A	1.9	2050	3.42	2.40	1.02	-0.11	-1.16	1.14
F127A	12.4	1050	2.13	2.09	0.047			
D210A	16.8	328	1.06	1.40	-0.34			
F127A/D210A	5.9	2400	2.85	2.58	0.27	-0.34	-0.91	0.56
Q138A	11	337	1.33	1.42	-0.092			
R119A	23.8	269	0.74	1.29	-0.55			
Q138A/R119A	2.47	4700	3.76	2.98	0.78	1.7	0.27	1.43
<i>C. Anticodon-G73 binding mutants</i>								
E188A	8.7	424	1.60	1.56	0.04			
S329A	12	120	0.66	0.81	-0.15			
E188A/S329A	3.23	2200	3.15	2.53	0.62	0.89	0.16	0.73
E188A	8.7	424	1.60	1.56	0.04			
T331A	5.7	200	1.40	1.12	0.28			
E188A/T331A	0.45	3500	4.60	2.80	1.80	1.6	0.12	1.48
F127A	12.4	1050	1.90	2.09	-0.19			
S329A	12	120	0.66	0.81	-0.15			
F127A/S329A	2	3000	3.60	2.70	0.90	1.04	-0.21	1.25
F127A	12.4	1050	1.90	2.09	-0.19			
T331A	5.7	200	1.40	1.12	0.28			
F127A/T331A	0.097	2850	5.40	2.60	2.80	2.10	-0.61	2.62

^a $\Delta\Delta G$ was calculated according to Carter et al. (1984):

$$\Delta\Delta G = -RT \ln \frac{k_{\text{cat}}/\text{Km (mutant)}}{k_{\text{cat}}/\text{Km (native)}}$$

^b $\Delta\Delta G_b$, variation of the binding free energy of the tRNA^{Asp}-AspRS system upon binding in the ground state

$$\Delta\Delta G_b = -RT \ln \frac{K_d (\text{mut})}{K_d (\text{native})}$$

^c $\Delta\Delta G k_{\text{cat}}$, variation of the activation free energy of k_{cat} : $\Delta\Delta G k_{\text{cat}} = \Delta\Delta G - \Delta\Delta G_b$

^d $D\Delta\Delta G = \Delta\Delta G$ of the multiple mutant ($\Delta\Delta G_{\text{exp.}}$) - $\Sigma \Delta\Delta G$ of the single mutants ($\Delta\Delta G_{\text{th.}}$). From Pütz et al. (1993).

^e $D\Delta\Delta G_b = \Delta\Delta G_b$ of the multiple mutant ($\Delta\Delta G_{\text{b,exp.}}$) - $\Sigma \Delta\Delta G_b$ of the single mutants ($\Delta\Delta G_{\text{b,th.}}$).

^f $D\Delta\Delta G k_{\text{cat}} = D\Delta\Delta G - D\Delta\Delta G_b$

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