Chapter 6
Amino acid Binding and
Mutational Studies on Pab-NTD
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6.1 Introduction

Pab-NTD sequence showed no significant homology to any other protein of known structure, however the coordinates when submitted to DALI-server revealed significant structural homology to that of D-tyr-tRNA^Tyr deacylase (DTD) from *E. coli*. This marked structural similarity at the monomeric level and similar dimerization propensity strongly indicate a common evolutionary origin for the Pab-NTD homologues in archaea and DTDs found in eubacteria and eukaryotes. Therefore, we set out to determine whether the structural similarity is also reflected in the functioning of both the protein families.

6.1.1 Amino acid Binding Studies on Pab-NTD

DTD has a wide substrate selectivity as its activity is not specific for a given tRNA. It could act on any tRNA which is aminoacylated with a D-amino acid, but not if it carries an L-amino acid, and removes it from the tRNA (Soutourina, *et al.*, 2004). Furthermore, the Pab-NTD-L-serine complex structure revealed that L-serine occupies only a small portion of a huge pocket, with a total surface area of 209 Å² filled with a number of water molecules. The strong structural resemblance and significant sequence conservation, prompted us to test whether Pab-NTD could also bind D-amino acids. Crystal soaking experiments were performed to find out the preferred amino acids for binding. Additionally, fluorescence-based approach was followed to confirm the binding of different amino acids to Pab-NTD.

6.1.1.1 Crystal Soaking Experiment

If a protein crystal is soaked in a solution containing its smaller interacting partner, they can enter the water channel of the crystal to reach the binding sites available in the protein molecule present in the crystal that are not masked by packing effects. The substrate binding induces movements of protein molecules within the crystal and can disturb the crystal lattice. It often results in a change of crystal appearance or sometimes even dissolution of crystal if the structural perturbation is huge. However, this can only be indicative and absence of any effect should not be considered as a clear case of lack of
interaction. To test the effect of D-amino acid solutions on the crystal, several soaking experiments were performed.

6.1.1.2 Fluorescence-based Binding Experiment

Fluorescence is a phenomenon where a molecule, called fluorescent molecule, absorbs light at a given wavelength and after a finite duration, it emits light of a different and generally longer wavelength. This fluorescence emission could be monitored and it provides insight into the structure, interactions, and dynamics of proteins. Much of the current research into protein and membrane dynamics has involved fluorescence spectroscopy of intrinsic biomolecular fluorescence as well as exogeneous protein or membrane specific fluorescent probes (Stryer, 1968). In protein molecules, specifically the aromatic residues like tyrosine and/or tryptophan, are responsible for intrinsic fluorescence. However, appropriate extrinsic fluorophores can be used in proteins lacking aromatic residues or intrinsic fluorescence. The most commonly used fluorophores for the protein dynamics are Aminonaphthalene sulphonate (ANS) and Bis-ANS.

The movements in intrinsic fluorescent proteins, upon binding the substrate, will either expose aromatic residues to the surface or result in the burying of few aromatic residues to the core of the protein. In both the cases, the intrinsic fluorescence spectra will change. Pab-NTD contained only one tryptophan (Trp 164) and that too in the disordered region, which could not be located in the electron density map. Hence, change in intrinsic fluorescence could not be monitored upon substrate binding.

The fluorophore ANS and Bis-ANS, when dissolved in water, emits only a very weak fluorescence. However, an immediate dramatic increase in the fluorescence is noted when these fluorophores binds to a protein molecule. Water molecules reduce the excited state energy of ANS and Bis-ANS hence lowers the fluorescent emission as well as quenching the fluorescence. The basis of the increased emission upon addition of the protein to ANS and Bis-ANS solution is due to the movement of the fluorophore from water to a hydrophobic environment on the protein surface. Any change in fluorescence, increase or decrease, after adding the ligand to the protein-ANS complex is a clear indication of ligand binding. Pab-NTD did not bind to ANS, instead an increased emission of Bis-ANS solution was observed after adding Pab-NTD to it. Thus, a Pab-
NCI – Bis-ANS complex was prepared and titrated with different amino acids to monitor their binding to the protein.

6.1.2 Mutational Studies on Pab-NTD

Pab-NTD could also bind to a variety of D-amino acids indicating a common evolutionary origin for DTDs and Pab-NTD homologs in archaea. Hence, it is possible that a primordial D-amino acid editing molecule was redesigned to bind to L-serine. In order to investigate this, mutational studies were performed on Pab-NTD. Based on L-serine selection mechanism, site-directed mutagenesis was done in Pab-NTD to check the possibility of selective abolition of binding of only one enantiomeric form in Pab-NTD, with the binding function retained intact for the other enantiomer. Two mutants K121M and F117A of Pab-NTD were created for binding studies. A similar fluorescence-based approach was utilized for amino acid binding experiments as done for Pab-NTD.

6.1.2.1 In vitro Site-directed Mutagenesis

In vitro site-directed mutagenesis is an invaluable technique for studying protein structure-function relationships, gene expression and for carrying out vector modification. In this technique, one can make point mutations, switch amino acids and delete or insert single or multiple amino acids. Site-directed mutagenesis was performed with the QuikChange™ XL site-directed kit (Stratagene) using recombinant plasmids of wild type as template and two synthetic oligonucleotide primers, each complementary to opposite strands of the vector, containing the desired mutation. The primers are annealed with the denatured plasmid during temperature cycling by PfuTurbo DNA polymerase, which replicates both plasmid strands with a high fidelity and without displacing the mutant primers. Incorporation of the primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with methylated and hemimethylated specific DNA endonuclease Dpn I that digest the parental DNA template and to select for mutation containing synthesized DNA. The nicked vector DNA containing the desired mutations is then transformed into XL1-Blue ultracompetent cells.
6.1.2.2 Cloning, Expression and Purification

Both mutants of Pab-NTD were tested for their expression levels. F117A could be expressed and purified in a similar way as that of the wild type protein (Section 1.2). On contrary, K121M could not be expressed. Since only 143 amino acids formed the structural core of Pab-NTD, the gene comprising of amino acids 1-143 (Pab-NTD) was PCR amplified and cloned as discussed (Section 1.1). This clone was further used as a template to produce K121M mutants.

Protein was grown in Luria-Bertani medium with 100 μg/ml ampicillin and overexpressed by inducing with IPTG in E. coli BL21(DE3) strain cells as described previously (Section 1.2). Two-step purification, Ni-NTA affinity column chromatography followed by gel filtration, was performed to obtain pure protein for binding assays. Expression and purification of Pab-NTD mutant was done using a similar protocol as used for the wild type protein.

6.1.2.3 Fluorescence-based Binding Experiments on Mutant Pab-NTD

The fluorescence-based binding experiment on Pab-NTD with 143 amino acid residues showed a similar binding property to various amino acids as that of the protein with 183 amino acid residues, except that it bound to ANS instead of Bis-ANS. Therefore, ANS fluorescence assay was used to monitor the binding of amino acids with Pab-NTD as well as its mutants.

This chapter contains information about crystal soaking and fluorescence-based binding experiments indicating that Pab-NTD could be a possible functional homolog of DTD. Based on the homology, a model for enforcement and perpetuation of homochirality during the early evolution of the translational apparatus has been proposed that underlines the essential role D-aminoacyl-tRNA deacylase might have played in forcing homochirality in proteins during early evolution. The chapter also incorporates mutational studies performed on Pab-NTD to test if the amino acid binding could be abolished or affected.
6.2 Materials and Methods

6.2.1 Materials

The synthetic oligonucleotide primers were custom synthesized by SIGMA. Expression vectors and restriction enzymes were used from Novagen and New England Biolabs (NEB) respectively. Site-directed mutagenesis was performed with the QuikChange™ XL site-directed kit (Stratagene). Mutations were confirmed by DNA sequencing. Cell cultures were grown in INNOVA 4330 refrigerated incubator shaker. Cells were lysed by sonication using a SONICS vibra-cell sonicator. SORVALL Revolution RC, using ESLA-1500 and SS34 rotor, was used for cell harvesting and pelleting respectively. HERAEUS Multifuge 3SR with HERAEUS S3334 rotor was used for concentration of protein. All the chromatographic steps were done at BIORAD BioLogic Duoflow FPLC system at room temperature with the columns and column matrix from Amersham Pharmacia. The fluorescence experiments were done using Hitachi F-4500 fluorescence spectrophotometer. The amino acids used for soaking and binding studies were from SIGMA-ALDRICH and all chemicals used for the experiments were of analytical grade.

6.2.2 Methods

6.2.2.1 Crystal Soaking

Initial attempts to obtain Pab-NTD structure in complex with D-amino acids, by soaking crystals in the D-amino acid solution mixed with Artificial Mother Liquor (AML) were not successful. AML is a solution with similar composition as that of the drop in which the crystals were grown. The soaking experiments damaged the crystals within few minutes so that no useful data could be collected from them. This damage could be thought of due to various reasons other than D-amino acid binding. Hence, to ensure that the crystals will not be damaged in AML, they were soaked in AML and they stayed intact for hours without any problem. For the experiments, crystals were soaked in the AML with various amino acids including L-threonine, L-serine, D-serine, D-threonine and D-tyrosine. Concentration of all the amino acids in the AML used for soaking experiment was 15 mM.
6.2.2.2 Fluorescence-based Binding Experiments

Bis-ANS fluorescence experiments were done to monitor the binding of amino acids with Pab-NTD (183 amino acids) as well as with FI17A Pab-NTD. An excitation wavelength 390 nm was used for Bis-ANS and all the spectra were recorded in the correct spectrum mode. Excitation and emission band passes were 5 nm each. Purified protein (0.1 mg/ml) and Bis-ANS (10 μM) mixture was titrated against 0 mM, 5 mM and 10 mM concentrations of L- and D-amino acids. Furthermore, ANS fluorescence assay was used to monitor the binding of amino acids with Pab-NTD (143 amino acids) as well as K121M Pab-NTD. An excitation wavelength of 380 nm was used for ANS and all the spectra were recorded in the correct spectrum mode. Excitation and emission band passes were 5 nm each. The purified protein (0.3 mg/ml) and ANS (100 μM) mixture was titrated against 0 mM, 5 mM and 10 mM concentrations of L- and D-amino acids.

6.2.2.3 Site-directed Mutagenesis in Pab-NTD

The Pab-NTD-L-serine complex structure revealed that there are quite a few conserved residues present around the binding site for L-serine and out of them two residues were considered for mutational studies. They were Lysi21, which was found to play a crucial role in L-serine selection, and Phe II7, which was found to cradle the substrate amino acid in the editing site. It is interesting to note here that a mutation of the corresponding lysine to alanine in \textit{M. jannaschii} ThrRS results in loss of editing activity (Beebe, \textit{et al.} 2004). This could be due to its inability to bind L-serine. Hence, two mutants K121M and F117A were created for Pab-NTD. Methionine was the best available structural replacement for keeping the hydrophobic interactions arising from the side chain of lysine and therefore it was the choice for replacement of lysine. Notably, structure based sequence alignment revealed that in all DTDs an invariant methionine was found at the corresponding position of Lysi21 in Pab-NTD.

Initially, the wild type clone of Pab-NTD (183 amino acids) without 6XHis tag was taken as the template DNA and synthetic oligonucleotide primers containing the desired mutation were designed for both mutants. The oligonucleotides used for the mutation K121M in Pab-NTD were forward primer 5' CCC TTC GGC TAT TAC ATG GCC TTT AAG ATA AGC 3' and reverse primer 5' GCT TAT CTT AAA GGC CAT.
GTA ATA GCC GAA GGG 3' and for F117A were forward primer 5' GGA AAG GCC CCC GCT GGC TAT TAG AAG 3' and reverse primer 5' CTT GTA ATA GCC AGC GGG GGC CTT TCC 3'. The nucleotides replaced from the wild-type gene are underlined. Both the mutations were confirmed by DNA sequencing. The clone for F117A mutant was then transformed into BL21(DE3), expressed and protein was purified in a similar way as that of wild type. However, K121M mutant, though being confirmed by DNA sequencing, could not be expressed when transformed in BL21(DE3). Since, only the first 143 residues could be identified in Pab-NTD structure hence, gene comprising of amino acids 1-143 (Pab-NTD) was PCR amplified by using the primers designed for 143 residues forward 5' GGAATT CCA TAT GAG GGT TCT CCT AAT CCA 3' and reverse 5' CCG CTC GAG CTG CCA TTC CTT TAT GGC GGC 3'. Additionally, the N-terminal methionine was found buried in the Pab-NTD structure thus the amplified product was cloned with C-terminal His tag into NdeI and XhoI sites of pET21b (Novagen) expression vector. The restriction sites for NdeI and XhoI have been indicated by * in forward and reverse primer, respectively. The C-Terminal His tagged Pab-NTD clone (143 amino acids) was taken as the template DNA for K121M mutation. This mutant could be expressed and purified for further studies.

6.2.2.4 Expression and Purification of Mutant Pab-NTD

Pab-NTD (143 amino acids) as well as both the mutants were expressed in Escherichia coli BL21(DE3) strain cells and the expression condition was similar to Pab-NTD (183 amino acids) as discussed in section 1.2. The pelleted cells were resuspended in ice-cold lysis buffer containing 50 mM Tris-HCl buffer pH 8.0, 150 mM NaCl and 10 mM Imidazole. Cells were lysed open by sonication. The crude cell extract was subjected to nucleic acid precipitation with 1% of streptomycin sulphate for 30 min at 277 K with constant stirring and was then centrifuged at 40000 x g (ss34 rotor) for 45 min at 277 K. The supernatant was loaded on Ni-NTA affinity column (Qiagen) previously equilibrated with lysis buffer. Protein was eluted with a buffer containing 50 mM Tris-HCl buffer pH 8.0, 150 mM NaCl and 100 mM Imidazole. The concentrated protein was further purified on a Superdex-75 gel filtration column in 50 mM Tris-HCl pH 8.0 and 50 mM NaCl. The purification of K121M was done using a similar protocol as followed for the Pab-NTD
(143 amino acids) whereas F117A mutant could be purified in a similar manner as that of Pab-NTD (183 amino acids).

6.3 Results and Discussion
6.3.1 D-amino acid Binding to Pab-NTD and its Mutants
6.3.1.1 Crystal Soaking Experiment

AML, which was tested to have no noticeable affect on the crystals, was mixed with various amino acid solutions and the crystal soaking was done. Initial soaking was done in AML with L-threonine, a negative control experiment, as L-threonine is not expected to bind to Pab-NTD. As expected in this soaking condition, no visible change in the external morphology of the crystal was observed and the crystal remained intact for hours. A positive control experiment was done by soaking Pab-NTD crystals in AML with L-serine and the crystals were dissolved in about 18 minutes. When soaked in AML along with D-serine, D-threonine or D-tyrosine, crystals were heavily damaged and dissolved within few minutes (Figure 6.1). Thus the soaking experiment indicated a clear preference of Pab-NTD for D-amino acids along with L-serine.
Figure 6.1 Crystal soaking experiment. (a) Crystal soaked in Artificial Mother Liquor (AML). (b) 90 min later. (c) Crystal immediately after soaking in AML with 15 mM L-serine solution. (d) 15 min later. (e) Crystal immediately after soaking in AML with 15 mM L-threonine. (f) 60 min later. (g) Crystal immediately after soaking in AML with 15 mM D-serine. (h) 21 min later. (i) Crystal immediately after transfer in AML with 15 mM D-threonine. (j) 9 min later. (k) Crystal in AML. (l) Crystal immediately after soaking in AML with 15 mM D-tyrosine.
### 6.3.1.2 Fluorescence-based Binding Experiment

Extrinsic fluorophores ANS and Bis-ANS were used to perform the fluorescence-based binding experiments. Amino acids used for fluorescence-based binding experiments for wild type and mutant Pab-NTD has been enlisted in table (Table 6.1).

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### Table 6.1 List of amino acids tested in fluorescence based binding experiments for wild type Pab-NTD as well as their mutants.

Bis-ANS was used for native Pab-NTD and its mutant F117A, both consisting of 183 amino acids and ANS was used for native Pab-NTD as well as K121M, both consisting of 143 amino acids. + indicates binding of amino acids to the protein and – indicates that amino acid did not show binding to the protein in the fluorescence-based binding experiment. Numbers in brackets denote the repetition of experiments in each case and the blank column indicates that the binding experiment was not done for that respective set of protein and amino acid.
6.3.2 Amino acid Binding with Native Pab-NTD

Pab-NTD–Bis-ANS complex was prepared to monitor the binding of different amino acids. The binding studies clearly showed a change in fluorescence intensity upon titration with L-serine (Figure 6.2a), used as a positive control, but did not show any change with L-threonine (Figure 6.2b), used as a negative control, to which Pab-NTD should not bind in accordance with its function. Interestingly, Pab-NTD showed binding to D-serine and D-threonine (Figure 6.2c, d). In fact, except for L-cysteine, several L-amino acids including glycine showed no binding. In contrast, of the 18 D-amino acids tested most of them showed change in the fluorescence intensity (Figure 6.7).
Figure 6.2. Amino acid–binding studies using Bis-ANS fluorescence. The change in fluorescence was monitored by titrating a premix of Pab-NTD (0.1 mg/ml) and Bis-ANS (10 μM) against (a) L-serine, (b) L-threonine, (c) D-serine and (d) D-threonine. Titration curves with 0 mM, 5 mM and 10 mM amino acid concentrations are shown. Arrows are indicating change in fluorescence intensity after adding amino acid to Pab-NTD and Bis-ANS premix.
6.3.3 Identification of a D-amino acid Binding Module in Archaea

The structure of DTD from *E. coli* is known, however, the mechanisms of amino acid selection and deacylation have not been experimentally studied (Ferri-Fioni, *et al.*, 2004). DTD homologs have been found in many eubacteria and eukaryotes including humans but they are conspicuously absent in archaea. Substantial structural homology between Pab-NTD and DTD, strongly indicated a common origin for DTDs and Pab-NTD homologs in archaea. D-amino acid binding studies were done on Pab-NTD to find out the possibility of whether Pab-NTD and DTD have functional commonality as well. Notably, crystal soaking experiments and fluorescence binding experiments indicated that despite a low sequence identity with DTD, Pab-NTD could bind to a variety of D-amino acids. These results indicated the presence of DTD-like fold in archaea. Thus, the study opens up a key evolutionary question regarding the presence of a D-amino acid binding module in all the three kingdoms of life, and its identification as a part of the translational apparatus in archaea.

6.3.4 Enforcing Homochirality During Translation

The efficiency of translational machinery lies in the selective incorporation of only L-amino acids during the formation of polypeptide chains. Thus, homochirality is a fundamental requirement of any efficient translational apparatus, which is ensured by stringent quality control mechanisms at different levels like selection of amino acids to be charged on to the tRNA, during formation of complex with elongation factor EF-Tu and at the ribosome (Agmon, *et al.*, 2004). It has been proposed that, after the initial fixation of homochirality by RNA molecules, primordial synthetases must have played a very crucial role in enforcing homochirality since they perform the first step of attaching an amino acid to the tRNA (Bailey, 1998; Tamura & Schimmel 2004). Some of the present day synthetases are found to face substrate discrimination problem and are reported to attach non-cognate amino acids to the tRNA. Such aaRSs possess editing modules that are covalently attached to them to remove these non-cognate substrates either as aminoacyl adenylate or as a part of the tRNA. Likewise, the primordial synthetases might have faced a crucial selection problem between L- and D-amino acids and could have been involved in the erroneous charging of tRNA with the opposite enantiomeric form,
D-amino acids. A protein made of only D-amino acids has been shown to be fully functional but with a reversal in chirality of ligand selection (Milton, et al., 1992). However, intermittent substitution of opposite chiral molecules is very likely to disrupt the secondary structural elements thus resulting in a non-functional protein (Mitchell, & Smith, 2003). Even if it were functional, practically it is an impossible task to replicate such a protein. Therefore during the early evolution of translational apparatus, a DTD-like protein attached to the translational apparatus might have played crucial role in enforcing homochirality.

A primordial weakly discriminating aaRS could have been coupled to DTD-like protein either covalently, like in this case, or functionally to remove the incorrectly charged D-amino acids on the tRNA (Figure 6.3). However, during the course of evolution, aaRSs might have gained significant specificity for L-amino acids and excluded D-amino acids from getting incorporated into polypeptide chains. Therefore, for most of the present day aaRSs, discrimination between the L-amino acids similar to its cognate amino acid is a more serious problem than discrimination against the opposite enantiomer. Hence, they could have gotten rid of the covalently linked DTD-like domain. Instead, they recruited an editing module to solve the discrimination problem of similar L-amino acids to ensure a faithful translation of the genetic code.
Figure 6.3 Proposed model for perpetuation of homochirality. A D-amino acid editing module covalently coupled to a primordial weakly discriminating synthetase must have ensured effective enforcement and perpetuation of homochirality in proteins.
Even though present day aaRSs are highly evolved, stereospecificity of some of them is not absolute. Few aaRSs eventually make errors by charging a D-amino acid on tRNA. It was first observed in *E. coli* and *B. subtilis* tyrosyl-tRNA synthetases (TyrRS) that they catalyze the formation of D-Tyr-tRNA\textsuperscript{Tyr} in vitro (Calender & Berg, 1966). Later, *S. cerevisiae* TyrRS (Soutourina, et al., 2000), as well as *E. coli* tryptophanyl-tRNA synthetase (TrpRS) and aspartyl-tRNA synthetases (AspRS), were also shown to catalyze the transfer of the D-enantiomer of their cognate amino acid to the corresponding tRNA species in vitro (Soutourina, et al., 2000). Hence, deacylases that specifically remove D-amino acids attached to different tRNAs were required and was first identified as a separate protein, which removes D-tyrosine from tRNA\textsuperscript{Tyr} in *E. coli* (Calender & Berg 1967).

### 6.3.5 Evolutionary Significance of a Covalently Attached DTD-like Protein to ThrRSs in Archaea

As for the presence of DTD-like protein found covalently attached to the archaeal ThrRS is concerned, there could be two possible evolutionary scenarios. The first possibility could be that archaeal ThrRS have been involved in frequent erroneous charging of its cognate tRNA with D-serine or D-threonine. Hence, there should be a requirement of a covalently linked DTD-like protein to rectify it. However, this hypothesis is ruled out as there has been no evidence for the abundance of D-amino acids in archaea. Furthermore, activation of D-amino acids in the catalytic site of ThrRS seems to be unlikely from a simple modeling study. The modeling suggests that even though an initial binding of D-threonine or D-serine is possible, due to the presence of zinc ion in the active site, the carboxylate will be placed in an unfavorable position to carry out the first step of the reaction i.e. the adenylate formation.

It is quite well established that serine and threonine as free amino acids can undergo spontaneous racemization upon heating and that their racemization rates increase depending on specific linkages like esterification (Neuberger, 1948; Takats, et al., 2003). Since charging of D-amino acids on the tRNA is unlikely through archaeal ThrRS, there might be a possibility of spontaneous racemization of already charged L-amino acids on tRNA due to the extreme environmental temperatures that they live in. Hence, most of
the present day archaeal ThrRSs might have preserved a Pab-NTD like domain in a covalently linked manner. This suggests that how effectively such D-amino acid deacylases coupled to the translational apparatus might have been involved in enforcement and perpetuation of homochirality in proteins.

6.3.6 Amino acid Binding in Mutant Pab-NTD

ANS fluorescence assay was used to monitor the binding of amino acids with Pab-NTD (143 amino acids) as well as K121M mutant of Pab-NTD. The binding studies on K121M clearly indicated a selective abolition of L-enantiomer binding (L-serine and L-cysteine) while preserving the D-enantiomeric binding (D-serine and D-cysteine) function of mutant Pab-NTD (Figure 6.4). As observed in the case of wild-type Pab-NTD, the K121M mutant could bind to a variety of D-amino acids like, D-threonine, D-tyrosine, D-aspartic acid and D-leucine (Figure 6.8).
Figure 6.4 Amino acid binding studies on Pab-NTD and its K121M mutant. The change in fluorescence is monitored by titrating the protein (0.3 mg/ml) – ANS (100 mM) premix against (a) and (b) L-serine, (c) and (d) D-serine, (e) and (f) L-cysteine, (g) and (h) D-cysteine, for Pab-NTD and K121M Pab-NTD, respectively. The curves with 0 mM, 5 mM, 10 mM and 15 mM of amino acid concentrations are shown, with arrows indicating the change in fluorescence intensity upon titration.
Bis-ANS fluorescence assay was used to monitor the binding of amino acids with F117A mutant of Pab-NTD. The fluorescence binding assay for F117A mutant of Pab-NTD also showed a complete abolition of L-serine binding as seen in K121M. In addition, on the contrary to K121M, it indicated abolition of binding to D-serine, D-threonine and D-tyrosine (Figure 6.5).
Figure 6.5 Amino acid binding studies on Pab-NTD and its F117A mutant. The change in fluorescence is monitored by titrating the protein F117A (0.3 mg/ml) – Bis-ANS (100 mM) premix against (a) L-serine, (b) L-threonine, (c) D-serine and (d) D-threonine, respectively. The curves with 0, 5 and 10 mM of amino acid concentrations are shown in green, red and cyan colours, respectively.
6.3.7 Selective Abolition of L-enantiomer Binding in Pab-NTD

The binding studies on K121M clearly indicated a selective abolition of L-enantiomer binding (L-serine and L-cysteine) by preserving the D-enantiomeric binding function of mutant Pab-NTD intact. As observed in the case of wild-type Pab-NTD, the K121M mutant could bind to a variety of D-amino acids like D-ser, D-thr, D-tyr, D-asp and D-leu. It is interesting to note here that a mutation of the corresponding lysine to alanine in *M. jannaschii* ThrRS resulted in a complete loss of editing activity, which could be linked to its inability to bind L-serine (Beebe, *et al.*, 2004). Thus, the study clearly demonstrates how nature might have introduced L-serine binding capacity in a canonical DTD like enzyme rather than using a different scaffold for L-serine removal as seen in eubacterial and eukaryotic ThrRSs.

6.3.8 Overlapping Binding Site in Pab-NTD Editing Pocket

The fluorescence binding assay for F117A mutant of Pab-NTD also showed a complete abolition of L-serine binding as seen in K121M. In addition, on the contrary to K121M, it also indicated abolition in binding of D-serine, D-threonine and D-tyrosine. This is more pronounced if one looks at the effect of mutations on the binding of D-tyrosine (Figure 6.6). These data taken together clearly indicate that L-serine and D-amino acids might share overlapping binding sites in the editing pocket of Pab-NTD. It could be a case where within a single pocket two different binding sites are available for two different ligands. However, this could be confirmed only by solving the structure of Pab-NTD in complex with D-amino acids.

Overall these results together indicate the identification of a D-amino acid deacylase-fold in all the three kingdom of life underlining an essential role it might have played in the enforcement and perpetuation of homochirality during the early evolution of the translational apparatus.
Figure 6.6 Affect of mutations on D-tyrosine binding. The change in fluorescence is monitored by titrating the protein (0.3 mg/ml) – ANS (100 mM) premix (a) Pab-NTD (143 amino acids) and (b) K121M mutant against D-tyrosine. The change in fluorescence was monitored by titrating the protein (0.3 mg/ml) – Bis-ANS (100 mM) premix (c) Pab-NTD (183 amino acids) and (d) F117A against D-tyrosine. The curves with 0, 5 and 10mM of amino acid concentrations are shown in green, red and cyan colour respectively, with arrows indicating the change in fluorescence intensity upon titration. The binding to D-tyrosine remains intact in K121M whereas, its completely lost in F117A, indicating an overlapping binding site for both the enantiomers.
Figure 6.7 Amino acid binding studies using Bis-ANS fluorescence. The change in fluorescence is monitored by titrating Pab-NTD (0.1 mg/ml) – Bis-ANS (10 μM) premix against (a) D-tyrosine, (b) L-tyrosine, (c) D-phenylalanine, (d) L-phenylalanine, (e) D-tryptophan, (f) L-tryptophan, (g) D-alanine (h) L-alanine, (i) D-valine, (j) L-valine, (k) D-isoleucine, (l) D-proline, (m) D-lysine, (n) D-methionine, (o) D-glutamic acid, (p) glycine, (q) D-asparagine, (r) D-glutamine, (s) D-arginine, (t) D-aspartic acid, (u) D-leucine, (v) L-cysteine, (w) D-histidine. The titration curves with 0 mM, 5 mM and 10 mM are shown in green, red and cyan respectively.
Figure 6.8 Amino acid binding studies on K121M-Pab-NTD using ANS fluorescence. The change in fluorescence is monitored by titrating K121M-Pab-NTD (0.3 mg/ml) – ANS (100 μM) premix against (a) L-threonine, (b) D-threonine, (c) L-tyrosine, (d) D-tyrosine, (e) L-aspartic acid, (f) D-aspartic acid, (g) L-leucine and (h) D-leucine. The titration curves with 0 mM, 5 mM, 10 mM and 15 mM are shown in green, red, cyan and blue respectively.