Unusual Role of Tryptophan residues in Structural and Functional properties of Mimi Virus TyrRS ($TyrRS_{apm}$)

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Abstract

In crystal structure, the homodimeric (α2) mimi-virus Tyrosyl-tRNA Synthetases (TyrRSapm) showed significant variation in dimer orientation as compared to other solved structures of TyrRSs. Previous report showed that Bacillus stearothermophilus N-terminal TyrRS exists as dimer under native condition and unfolds through a monomeric intermediate. In our previous studies, we have reported that the C-terminal anticodon binding domain of TyrRSapm might have an indirect role in noncanonical dimer formation. To study the implication of the non-canonical dimer interface on the structural and functional organization of TyrRSapm, we replaced all three W residues (both of dimer interface and C-terminal domain) systematically with F residues (using site directed mutagenesis) with four different combinations (W80F/W120F, W278, W80F/W278F, W120F/W278F). We took advantage of Trp (W) fluorescence as the biophysical reporter for probing dimer interface study. The circular dichroism (CD) spectra of Trp mutants are dramatically different from wild-type indicating a huge conformation reorientation due to mutations. Another unusual feature of this enzyme is that it contains 10 cysteine residues per monomer and none of them involved in disulphide bridge formation. Using DTNB reaction as a probe again it was observed that mutants behave differently indicating a major perturbation. Fluorescence anisotropy supported by analytical ultracentrifugation data showed that TyrRSapm and its mutants exist as dimer. The unfolding pattern of N-terminal and C-terminal domain shows significant difference from each other indicating differential melting of domains. Aminoacylation reaction showed that two of the four Trp. mutants retain enzyme activities though different as compared to WT, while the other two mutants lost their activities. Hence it can be concluded that, the Trp residues play a crucial role in the structural as well as functional organization of TyrRSapm that may be reflected as the non canonical orientation of dimer conformation in crystal structure.

Key Words : Mimivirus, Tyrosyl-tRNA Synthetase, Fluorescence, Unfolding, Circular Dichroism, nalytical Ultracentrifuge

Introduction:

Tyrosyl-tRNA synthetases belong to class I c of aaRSs, together with TrpRS; both are homodimers, a feature generally shared by most of class II synthetases [1]. TyrRSs are all comprises similarly, with an N-terminal catalytic domain, having the connective peptide 1 (CP1) region responsible for dimerization, followed by a C-terminal domain [1] . However, important differences in sequence as well as in architecture are observed at the C-terminal domains of TyrRSs, such as archaeal TyrRSs are encoded by the shortest, whereas vertebrate TyrRSs exhibit a large extra C-terminal domain [1].

Surprisingly, TyrRS is also encoded in genome of the second largest DNA virus Acanthamoeba polyphega (Mimi virus). The mimic virus has defined a new boundary between viruses and parasitic cellular organisms as its genome exhibits numerous genes never encountered before in any other viruses; for instances genes corresponding to central

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components of the protein translation machinery, an exclusive signature of cellular organisms [2,3]. The genome of A. polyphega contains four aaRSs namely MetRS, TyrRS, ArgRS, and CysRS. Among them, the crystal structure of tyrosyl-tRNA synthetase (TyrRSapm) has been solved recently [2]. TyrRSapm exhibits the typical fold of the TyrRS core domain and with an N-terminal Rossmann-fold catalytic domain, an anticodon binding domain, and no extra C-terminal domain [2]. Mimi virus TyrRS (TyrRSapm) is a homodimer (α2) and exhibits some unique features [2], most notable is the dimeric interface. When one monomer of TyrRSapm is superimposed on the corresponding monomer of other solved crystal structure of TyrRSs having canonical dimeric interface, the second monomer lies nearly at 90^0 angle relative to its position in the crystal of other dimeric proteins [2].

Till date, The Bacillus stearothermophilus TyrRS (BstTyrRS) is the most extensively characterize, both biochemical and biophysically. It was reported by Bedouelle and its group that Bacillus stearothermophilus N-terminal TyrRS exists as dimer under native condition and unfolds through a monomeric intermediate [4]. However, in the previous studies we have reported that the isolated N-terminal containing entire CP1 domain, responsible for the dimerization of TyrRSapm, exists as monomer in solution. We argued that the C-terminal anticodon binding domain of TyrRSapm might have an indirect role in dimer formation [5].

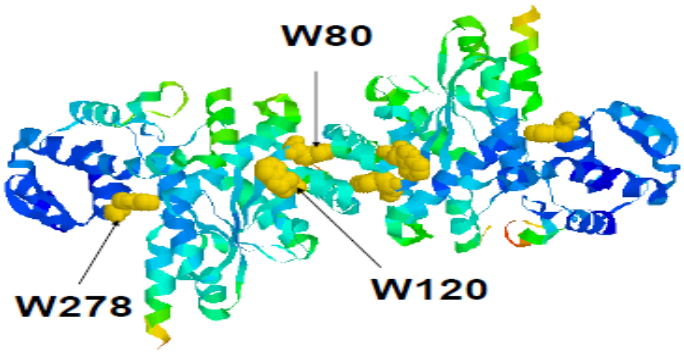


Fig.1. Schematic diagram showing the positions of Tryptophan (W) residues of TyrRSapm [pdb id 2J5B. Abergel, C., Rudinger-Thirion, J., Giege, R., Claverie, J.M. (2007) J.Virol. 81: 12406]

The non-canonical dimeric interface organization due to unusual CP1 domain [2], indirect role of the C-terminal domain in non-canonical dimer formation [5] and speculated probable "4th domain of life" [6], all these idiosyncratic features evoked the question whether the non-canonical dimer interface has any implication on the structural and functional organization of TyrRSapm.

TyrRSapm has three tryptophan residues (W80, W120 and W278). Two of them (W80 and W120) are present at the dimer interface and the other one (W278) is present at the C-terminal domain, away from dimeric interface [2] (Fig.1). Using site directed mutagenesis (SDM), we replaced all three W residues (both of dimer interface and C-terminal domain) systematically with F residues with four different combinations (W80F/ W120F, W278, W80F/W278F, W120F/W278F). We took advantage of Trp (W) fluorescence as the biophysical reporter for probing dimer interface study.

Here we report very unusual structural and function role of each W residues. We found that the circular dichroism (CD) spectra of W mutants are dramatically different from wild-type indicating a huge conformation reorientation due to replacement. Using DTNB reaction as a probe, it was observed that mutants behave differently, further indicating a major structural perturbation. The unfolding pattern of N-terminal and C-terminal domain shows significant difference from each other indicating differential melting of domains. Aminoacylation assay indicated that two of the four W mutants retain enzyme activities, while the other two mutants completely inactive. Hence we concluded that the W residues play a crucial role in the structural as well as functional organization of TyrRSapm and the interfacial W residues (W80 and W120) may play critical role for maintaining non canonical orientation of dimer conformation in crystal structure.

Materials and Methods:

Materials

The clone of full-length Mimivirus TyrRS (TyrRSapm) was a gift from Professor Chantal Abergel (Information Génomique etStructurale, CNRS – IBSM, Marseille, France). All the chemicals used were of molecular biology grade from Sigma, HI-MEDIA, E-Merck and SRL. Primers were obtained from Sigma Chemical Company. Plasmid containing the human tRNATyr gene was a gift from Dr. Eric First (Louisiana State University Health Sciences Center-School of Medicine in Shreveport). Fluorescein isothiocyanate (FITC) was kindly provided by Professor David Jameson, University of Hawaii.

Protein isolation and purification

TyrRSapm contains three tryptophans residues (W), two residues are at dimer interface (i.e, N-terminal domain, W80 and W120) and remaining one is at C-terminal domain (W278). Point mutations were introduced by Site Directed Mutagenesis (SDM) to construct W278F (W80, W120), W80F/W120F (W278), W80F/W278F (W120) and W120F/W278F (W80) mutants, following the Quick Change procedure (Stratagene). The protein purification was done by a published protocol [1, 2].

Fluorometric studies

All fluorescence measurements were performed in a HITACHI F-7000 fluorescence spectrophotometer using a quartz cuvette of 1 cm path length. The protein (4 μM) was incubated in the desired concentration of GdnHCl, urea and different pH buffers for 18-24 hours at 25ºC to attain thermodynamic equilibrium. The GdnHCl and urea induced equilibrium unfolding was carried out in 20 mM Tris-HCl buffer, pH 7.4. All the buffer solutions of different pH values were prepared according to the standard procedure [7]. Tryptophan was selectively excited at 295 nm. Both excitation and emission slit widths were set at 5 nm for all experiments. Measurement of ANS fluorescence was carried out to observe the extent of hydrophobic surfaces in the proteins. Protein (4 μM) was incubated with almost 8 molar excess of ANS for more than 30 minitues. The excitation wave length was set to 420 nm and intensities were recorded at 482 nm. Intensity of the buffer without protein (blank) was always subtracted.

Cysteine reactivity studies

A 40 mM stock solution of DTNB was prepared by weight in 20 mM Tris–HCl (pH 7.4) buffer containing 1 mM EDTA. Molar concentration of the stock DTNB solution was determined by measuring absorbance at 324 nm (molar extinction coefficient at 324 nm = 19800 M-1 cm-1). For reactive cysteine estimation, absorbance was recorded at 412 nm in Shimadzu UV-2450 Spectrophotometer. Protein samples (4 µM) were mixed with excess concentration of DTNB (2 mM) to ensure complete reaction of DTNB with free cysteine.

Acrylamide quenching studies

Acrylamide quenching studies were carried out to explore tryptophan accessibilities. Spectra were measured in 20 mM Tris-HCl (pH 7.4). Protein concentrations were kept at 8 µM. The acrylamide was added gradually from 1 M stock solution. For a single class of tryptophan, quenching data were analyzed in terms of the linear Stern-Volmer equation:

$$F0/F = 1+Ksv\,[Q]$$

Where F0 and F are fluorescence intensities in the absence and presence of the quencher respectively, Ksv is the Stern-Volmer constant and [Q] is the molar concentration of the quencher. For two classes of tryptophan the data was fitted to a modified Stern-Volmer equation [8].

$$F_0/F = 1 + \frac{(f_aK_a + f_bK_b + K_aK_b\,[Q])\,[Q]}{1 + (f_aK_b + f_bK_a)\,[Q]} \times e^{V\,[Q]}$$

Where fi, Ki, Vi and [Q] are fraction of the total fluorescence corresponding to the ith tryptophan at a given wavelength, collisional and static quenching constants, and molar concentration of the quencher respectively. Eftink and Ghiron [9] also showed that, for a system having more than one class of fluorophores, the initial slope of an F0/F plot will be approximately equal to ΣfiKi, the weighted average of the individual quenching constants, referred to as the effective Stern-Volmer constant, Ksv (eff).

Lifetime measurements

Fluorescence lifetimes were determined in a nanosecond time-domain fluorometer operated in the time-correlated single-photon-counting mode. Measurements were determined from the total emission intensity decays of 50 µM protein samples in 20 mM Tris-HCl (pH 7.4). The N2 -emission line at 303 nm was used to excite Trp, while the emission was monitored at 340 nm. The lifetimes were obtained from decay curves by fitting to a three-exponential function after deconvoluting the lamp response function.

Fluorophore labeling of TyrRSapm

For the covalent labeling of TyrRSapm and its W variants with fluorescein isothiocyanate (FITC), a required amount of a concentrated stock solution (3.6 mM) of the probe was added to a 50 μM TyrRSapm solution in 100 mM sodium carbonate-bicarbonate buffer (pH 9) to attain final enzyme:probe molar ratio of 1:10. The mixture was incubated overnight at 4ºC, followed by termination of the reaction by addition of 10 mM β-Mercaptoethanol. The labeled protein was separated from unbound dye after passing through Sephadex G-25 column equilibrated with the same buffer. The eluted fractions containing labeled protein were pooled and dialyzed extensively for 24 hours with five changes in 20 mM Tris-HCl (pH 7.4) buffer containing 16 mM MgCl2 to get rid of excess unbound fluorophores. The labeling efficiency was assessed spectrophotemetrically and incorporation of probe to protein (F/P) was found to be ~ 1:1. Necessary corrections were performed to negate the contribution of the probes in absorbance at 280 nm.

Steady State Anisotropy

FITC labeled proteins were excited at 490 nm and emission was recorded at 525 nm. Labeled protein samples were serially diluted from 4 μM to ~ 2 nM concentration with 20 mM Tris-HCl (pH 7.4) buffer containing 16 mM MgCl2 For the samples saturated with ligands, 1 mM of L-tyrosine, 2 mM of ATP and 5 μM of tRNATyr separately or together was first incubated with the enzyme sample for 30 min at 25 0C. While measuring the anisotropy values at ligand bound condition, serial dilutions were carried out with the buffer in presence of respective ligand at identical concentration, to maintain an unchanged ligand concentration of the sample throughout the experiment. For all experiments, the excitation and emission band pass were 5 nm. Measurements were made by rotating emission polarizer at regular interval to obtain perpendicular ($I^{\perp}$) and parallel ($I^{\parallel}$) decay components. The diffraction grating factor G and anisotropy value A were obtained as follows:

$$G = I^{\perp} / I^{\parallel} \quad A = (I^{\parallel} - G.I^{\perp}) / (I^{\parallel} + 2.G.I^{\perp})$$

The dissociation constants were calculated using the method described elsewhere using Kyplot [10, 11].

Circular Dichroism (CD) studies

CD spectra were recorded on a JASCO J815 spectropolarimeter, using 20 mM Tris-HCl (pH 7.4) containing GdnHCl to the desired concentration when required , or other buffers of different pH .For secondary structure estimation, 5μM protein samples were monitored in Far UV region (200-260 nm) using 1 mm path length cuvette. Changes in the tertiary structure protein samples (20 μM) in same buffer were monitored at Near UV region (250-350 nm) using 10 mm path length cuvette. The mutants and the wild type protein concentrations were first determined by measuring the absorbance at 280 nm and the concentrations were appropriately adjusted by measuring the SDS-PAGE band intensities using Image Analysis Software Quantity One. α-helix, β-strand and random coil percentage were calculated using CDNN, a neural-network approach (G. Bohm).

Guanidine Hydrochloride (GdnHCl) induced unfolding studies

GdnHCl induced unfolding of WT and W mutants of TyrRSapm was performed with increasing concentration of denaturants. 4µM protein samples were incubated in the desired concentration of GdnHCl for 18-24 hours at 25ºC to attain thermodynamic equilibrium. Equilibrium unfolding was carried out in 20 mM Tris-HCl buffer, pH 7.4.

Acid Denaturation

Acid denaturation of WT and W mutants was carried out as a function of pH using KCl-HCl (pH 1- 2), Gly-HCl (pH 2.5, 3, 10.5, 11, 12.5), Tris-HCl (pH 7.4, 8, 8.5, 9.5, 10) and Sodium acetate (pH 4-5). 4µM protein samples were incubated for 18 to 24 hours at 25ºC with the buffers of desired pH to ensure thermodynamic equilibrium.

1,8-anilino-sulphonate (**ANS**) **binding** studies

Measurement of ANS fluorescence was carried out to observe the extent of hydrophobic surfaces in the proteins. Protein samples were incubated with 8 molar excess of ANS for more than 30 minutes in dark. The excitation wave length was set to 420 nm and intensities were recorded at 482 nm. Emission spectra were collected between 450 nm to 550 nm. Intensity of the buffer without protein (blank) was always subtracted.

Analytical Ultracentrifugation

Sedimentation Equilibrium experiments were performed in a Beckmann XL1-optima ultracentrifuge at 4 0C (Beckman-Coulter Instruments) using An50-Ti rotor. To avoid any suspended contaminants, protein samples as well as the reference buffer solutions were centrifuged at 5000 rpm for 4 min at room temperature in a tabletop centrifuge before the experiments. 110 µl of each protein sample was loaded against 120 µl buffer solutions in a 12 mm charcoal filled six-chambered EPON centerpiece. TyrRSapm was centrifuged at 9000 rpm for 70 hrs and at 12000 rpm for the next 12 hrs, During the entire experiment, protein samples were kept in 20 mM Tris-HCl (pH 7.4) buffer containing 100 mM NaCl. Data were recorded at 220 nm as a function of radial position at the three loading concentrations of 0.2 µM, 0.5 µM and 2 µM for TyrRSapm. Solution density was calculated at 40C using Sednterp program from buffer composition as 1.00733 g ml-1 [11]. The partial specific volumes of proteins was calculated at 40C as 0.7321 ml g-1 for TyrRSapm from the respective amino acid composition using Sednterp. The data were analyzed by global nonlinear least squares fitting using SEDPHAT software (v10. 4, 2012) [11]. TyrRSapm data was fitted in monomer-dimer and monomer-dimer-tetramer model to obtain both molecular weight and dissociation constant. During fitting, both molecular weight and dissociation constant values were allowed to float. Molecular weight (Mr) and dissociation constants were obtained by a model-dependent method with least chi-square and least rmsd value.

Results:

The emission spectra of mutants are different

Tryptophan fluorescence is widely used as reporter of protein local environment [7]. The emission maxima of WT, W278F, W80F/W120F, W80F/W278F and W120F/W278F under

native condition are 339 nm, 335.8 nm, 336.4 nm, 335.2 nm and 335.2 nm respectively. The fluorescence intensities of the mutants are substantially different from WT either due to the presence of fewer tryptophans than WT or decreased quantum yield. The summation of fluorescence intensity of equal concentration protein in different permutations such as showed

(W278F + W80F/W120F or W80F/W120F + W80F/W278F + W120F/W278F)

intensity of WT is much higher than any of the mutant combinations [9,12] (Fig.2). From the figure 1, it can be easily observed that W80 and W120 are present at the dimer interface and either of these W residues are surrounded by 3 other W residues (10, 10 and 25 Å, one on the same chain and the other two at the dimeric interface of the other monomer), thus there will be substantial FRET one would expect. However, absence of FRET in mutants should be manifested by increase in fluorescence quantum yield. The reverse trend as observed here that addition of any combination give far below value than WT indicated a possible perturbation in conformation around W residues that require further investigations.

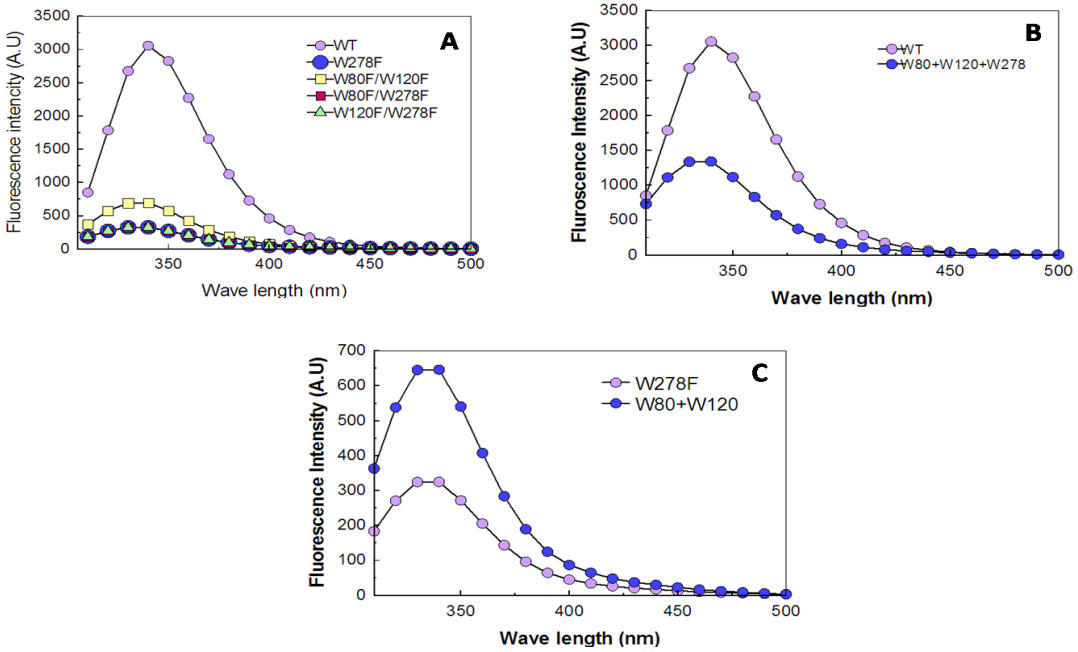


Fig.2. Additivity of Emission Spectra of WT and W mutants of TyrRSapm. A. Emission Spectra of WT and all W mutants. B. WT emission spectra and added emission spectra of all single W mutants. C. Emission spectra of W278F (W80, W120) and added emission spectra of W120F/W278F (W80) and W80F/W278F (W120F). The protein concentrations were 4 µM per monomer. All the protein samples were in 20 mM Tris-HCl buffer, pH 7.4. All the samples were excited at 295 nm. Both the excitation and emission band passes were 5 nm.

Accessibility of W residues is dissimilar in mutants

Protein tryptophan fluorescence is efficiently quenched by neutral quencher acrylamide and routinely used to measure the degree of exposure of tryptophanyl moieties in proteins (Eftink and Ghiron 1976 biochem). Linear Stern-Volmer plot is an indication of a single class of fluorophores, which are equally accessible to quencher [8]. Non-linear Stern-Volmer plots may also occur in the case of purely collisional quenching, where some of the fluorophores are

less accessible than others. [13]. The Stern-Volmer constant (Ksv) values as determined from Stern-Volmer plot (Fig 3) of WT, W80F/W120F, W278F, W80F/W278F and W120F/W278F are given in Table 1. Surprisingly, WT have the highest Stern-Volmer constant, i.e., all the tryptophans are less accessible in mutants than WT. If the structure remained unperturbed after mutation, one would expect Ksv value of WT must be intermediate between two mutants. Thus acrylamide quenching experiment further support that there is structural perturbation around tryptophan as observed in fluorescence emission spectra [14, 15].

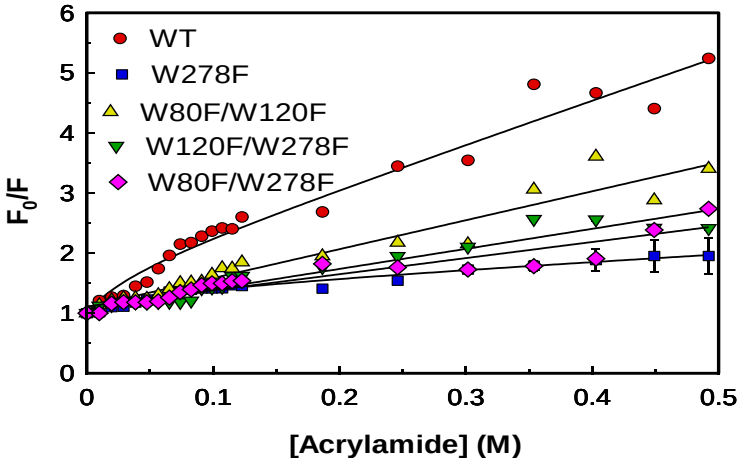


Fig.3: Acrylamide quenching of WT and Trp. mutants of TyrRSapm. Protein concentration was 8µM per monomer and all of the samples were in 20 mM Tris-HCl buffer, pH 7.4. Excitation wave length was 295 nm and both excitation and emission band pass ware 5 nm. The experiment was done using HITACHI F-7000 spectrofluorometer.

The experiment was done using HITACHI F-7000 spectro-fluorometer. The excitation wavelength 295nm, emission wavelength 340 nm, and bandpasses 5 nm for both excitation and emission. The protein concentration was 4 µM. The buffer used was 20mM Tris-HCl, pH 7.4. The experiment was carried out at 25ºC.

Table: 1. Sterm-Volmer constant under native condition.

	WT	W278F	W80F/W120F	W120F/W278F	W80F/W278F
Ksv(1)M-1	21.99±1.02	6.68±0.5	4.81±1.14	3.32±1.11	2.68±1.2
fa	0.41±0.02	0.19±0.02	-	-	-
Ksv(2)M-1	1.14±0.03	2.93±0.04	-	-	-
fb	0.58±0.02	0.8±0.03	-	-	-

The structural perturbation in mutants was observed both at secondary and tertiary level

Far-UV CD is used routinely to determine the secondary structure content of a protein [16]. Each protein generally has characteristics CD profile between 200-250nm ranges. The WT

TyrRSapm exhibited large negative ellipticity at 222 and 208nm showing high α-helix content. When the data fitted in CDNN program, the α-helix content corroborated well with crystal structure. To our surprise, we found major changes in secondary structure in all mutants with α-helical content ranges from ~ 36% to ~ 80% for W80F/W120F and W278F respectively (Table 2, Fig. 4). Though W residues contributes in the range between 200-250 nm, thus making the absolute prediction in secondary structural composition difficult, but it may be concluded that there are substantial changes in secondary structure due to mutations. Also there are some reports regarding exciton coupling between two W residues that can influence CD spectrum in this range [16]. The results showed no such characteristics spectra ruling out the possibilities of exciton coupling.

The near UV CD spectra (between 250-320 nm) generally contributed by aromatic side chains (Trp, Tyr and Phe) and disulphide bonds [17]. As clear from crystal structure of TyrRSapm that none of cysteine residues are involved in disulphide bridges [2], the spectra obtained are largely contributed by the aromatic residues. The WT showed nearly flat spectra near baseline throughout the region while all the other mutants have positive contribution indicating a more rigid environment around aromatic residues in mutants [17]. Such a change in environment can only be explained only if the surrounding residues around W reorient themselves [17].

Table: 2. α-helix, β-strand and random coil percentage of WT and W mutants of TyrRSapm using CDNN program. Protein concentrations were 10 µM per monomer. The buffer used was 20 mM tris-HCl, pH 7.4. The data was recorded from 200 nm to 250 nm.

TyrRSapm	% of α helix	% of β sheet	% of Random coil
Wt	48	Ant.parallel-4.1,Parallell-5.2, beta turn-13.3	29.5
W278F	79.2	A.P-1.6,P-2.3, B.turn-9.7	12.5
W80F/W120F	40	A.P.-6.5,P.-7.7,B.turn-15.4	31
W120F/W278F	57.1	A.P.-3.7,P-5,B-turn- 12.7	23.4
W80F/W278F	35.7	A.P-7.5,P-9.2,B-turn-16	36

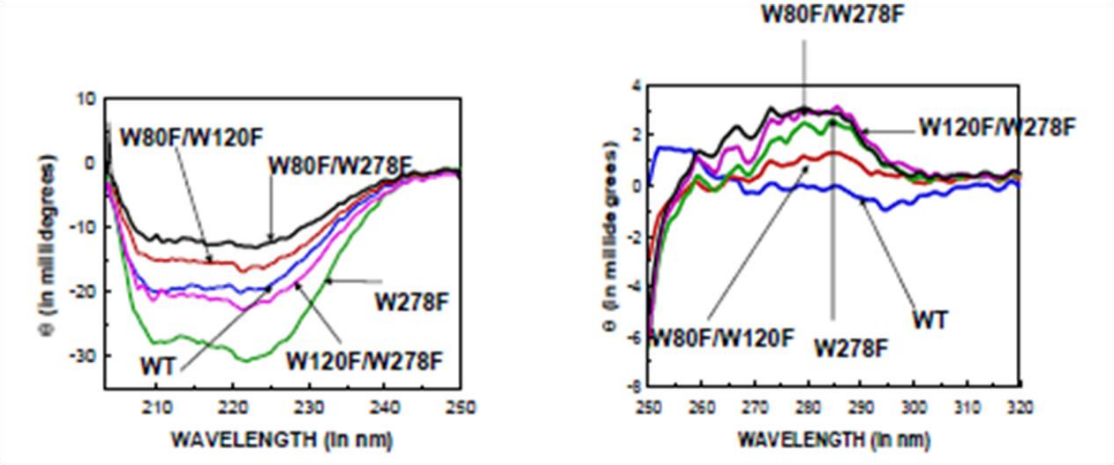


Fig. 4. A. Far UV-CD of WT and all Trp. mutants of TyrRSapm. B. Near UV-CD of WT and all Trp. mutants of TyrRSapm.

For far UV CD experiments, protein concentrations were 10 µM per monomer and for near UV CD experiment, protein concentrations were 40 µM per monomer. All the samples were in 20 mM tris-HCl buffer, pH 7.4. The data were taken from 200 nm to 250 nm in a 1-mm path-length cuvette, with 1 nm of bandwidth and 5 s of averaging time. Five scans were taken and averaged for each sample. Protein-only spectra were obtained by subtracting the CD signal from the corresponding buffer.

Mutants showed widely varied accessibility of cysteine residues

The Ellman's Reagent or DTNB (5, 5'-dithiobis-(2-nitrobenzoic acid) is frequently used for determination of free sulfhydryl groups in proteins. Formation of 5-thiolata-2-nitrobenzoate by DTNB reaction with free sulfhydryl group has a characteristic yellow color with absorption peak at 412 nm [18, 19]. TyrRSapm has unusually large number of cysteine residue (10 per monomer), but none of them form disulphide bond in crystal structure [2].To ensure that the decreased quantum yield and CD spectral changes of the W mutants of TyrRSapm are due to structural perturbations, the DTNB reaction was performed with WT and all the mutants [20]. Our result showed that WT TyrRSapm has only three reactive cysteine residues, W80F/W120F mutant have no free reactive cysteine, W278F mutant have all the 10 cysteine residues per monomer were accessible, W80F/W278F mutant have one and W120F/W278F mutant have two reactive cysteine residues. Hence the accessibility of cysteine residues of W mutants of TyrRSapm varied widely (Fig. 5, Table 4). This result clearly strengthen our claim that the flurescence spectral changes, or changes in far- and near-UV are due to substantial structural perturbations.

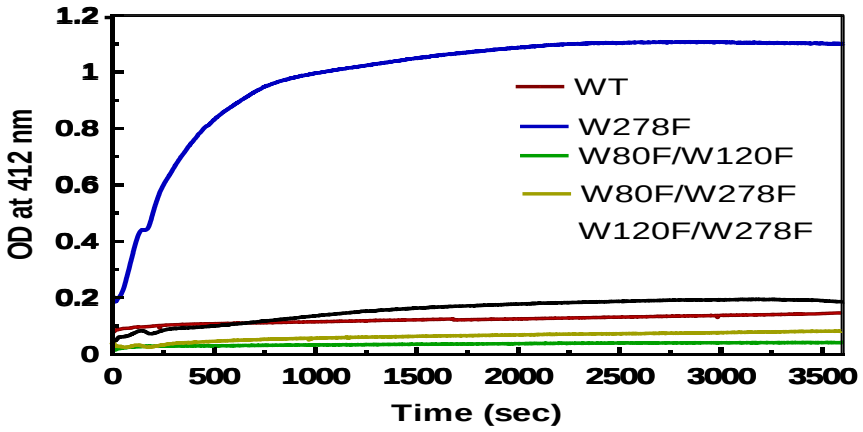


Fig. 5. DTNB reaction of WT and Trp. mutants of TyrRSapm. Protein concentrations were 4 µM. All the protein samples were in 20 mM Tris-HCl buffer, pH 7.4. Changes in absorbance at 412 nm due to the release of the product 5-thiolata-2- nitrobenzoate were recorded in a Shimadzu UV-2450 spectrophotometer.

Table 4: Number of reactive cysteine of WT and W mutants of TyrRSapm.

	WT	W278F	W80F/W120F	W80F/W278F	W120F/W278F
No. of Reactive Cysteine	3	20	0	1	2

Changes in absorbance at 412 nm due to the release of the product 5-thiolata-2-nitrobenzoate were recorded in a Shimadzu UV-2450 spectrophotometer. The number of reactive cysteines was calculated using a molar extinction coefficient of 14150 M_1 cm_1 for native conditions.

Long range domain-domain communication may be operative in TyrRSapm

Intrinsic tryptophan lifetime measurement helps in identifying different conformations of protein [15]. It provides information about the molecular microenvironment of a fluorescent molecule [15] Lifetime of a fluorophore can be modified by depletion of the excited state due to the factors such as ionic strength, hydrophobicity, oxygen concentration, binding to macromolecules, and the proximity of molecules (FRET). Lifetime measurements thus can be used as indicators of these parameters [14]. These measurements are generally absolute and independent of the fluorophore concentration [14], however dependent on tryptophan microenvironment. The tryptophan lifetime of WT and all the W variants were determined. Notably, WT showed triple exponential decay. The average lifetime values (<τ>) of WT, W278F, W80F/W120F, W80F/W278F and W120F/W278F are 4.26, 1.12, 1.03, 1.46 and 1.15 ns respectively (Table 3). According to the rule of additivity, combination of lifetime values of all the three single Tryptophan mutants did not equal the value of lifetime of WT protein [21]. Combination of lifetime values of W80 and W120 also did not reproduce the lifetime value of W278F either. This absence of additivity meant either there are energy transfers between tryptophan residues or conformational rearrangement around W residues [21]. The average lifetime decreased significantly with removal of any of W resides and happened mainly due to large decrease in longer lifetime component for all the mutants. Thus for mutants one may consider the W lifetime decay essentially as biexponential with long life-time exponent is almost abolished. [16]. Furthermore, it is known that the amino acids present around the tryptophan indole ring plays a major role influencing the life time of tryptophans [22]. Though the crystal structure of the WT TyrRSapm is available, substantial structural reorientation due to mutation as probed by other experiments described above making it difficult to predict how the surrounding amino acid residues affecting average lifetime of tryptophan. The results indicated that any mutating one W residue affecting the other two W residues, this is not surprising for W80 and W120 as they are close to each other, however W278 apparently far apart from both W80 and W120. This may only be explained by long-range domain-domain communication which is common in many aaRSs [22-26].

Table: 3 Fluorescence life time values of TyrRSapm and its varients.

TyrRSapm	τ (1,2 and3)	α (1, 2 and 3)	<τ> ns
WT	0.51, 2.06, 8.04	0.37, 0.16, 0.46	4.26
W278F(W80 and W120)	0.75, 2.14, 5.9	0.74, 0.25, 0.003	1.12
W80F/W120F (W 278)	0.59, 1.89, 5.95	0.68, 0.31, 0.007	1.03
W80F/W278F (W 120)	0.7, 1.92, 5.37	0.7, 0.29, 0.0038	1.46
W120F/W278F (W 80)	0.78, 2.08, 5.97	0.72, 0.27, 0.004	1.15

Lifetimes were determined in a nanosecond time-domain fluorometer operated in the time-correlated single-photon-counting mode. Measurements were determined from the total emission intensity decays of 50 µM protein samples in 20 mM Tris-HCl (pH 7.4).

The Fluorescence of WT protein is dominated by W278

Guanidium hydrochloride (GdnHCl) is considered as one of the strongest denaturant of proteins. At increasing concentration of GdnHCl, protein lost its folded structure, become random coil and thus gradually shifted W residues to more polar environment causing red shift of the emission maxima [27, 28]. Changes in CD signal and flurorescence intensity at 340 nm (Fig. 6) (F340) as a function of GdnHCl concentration revealed some interesting features. The CD signal changes at 222 nm did not follow any particular pattern and change randomly. One observation is common at low concentration of denaturant, all the mutants along with WT lost significant signal at 222 nm indicating loss of alpha-helix [30]. At concentration greater than 1 M denaturant, they regained ellipticity and finally lost at higher denaturant concentration (fig 6). Further investigation revealed that the behavior of W278F and W80F/W120F are similar, while W80F/W278F and W120F/W278F were shown similar pattern (Fig.6) with increasing concentration of GdnHCl. Similar changes in fluorescent intensity (Fig. 6) and emission maxima (Fig. 5) for WT and W80F/W120F may be concluded as WT protein fluorescence is dominated by W278 fluorescence and fluorescence maxima at 346 nm of W278F at 7.5 M GdnHCl clearly indicated that the N-terminal domain more resistant to denaturant (Fig.5). The nature of the curve of WT and W80F/W120F is very unusual up to 2 M GdnHCl from the rest, while at higher concentration they behave more consistently as there was gradual red shift of emission maxima with increasing concentration of GdnHCl (Fig. 5). However a trouble was faced while fitting the data. A poor fitting was observed with either three state or two state model, due to the unusual emission maxima of WT and W80F/W120F mutant with increasing concentration of GdnHCl up to 2M (Fig.5). Hence the data was fitted from 2-7.5 M GdnHCl assuming two-state model (Table 5).

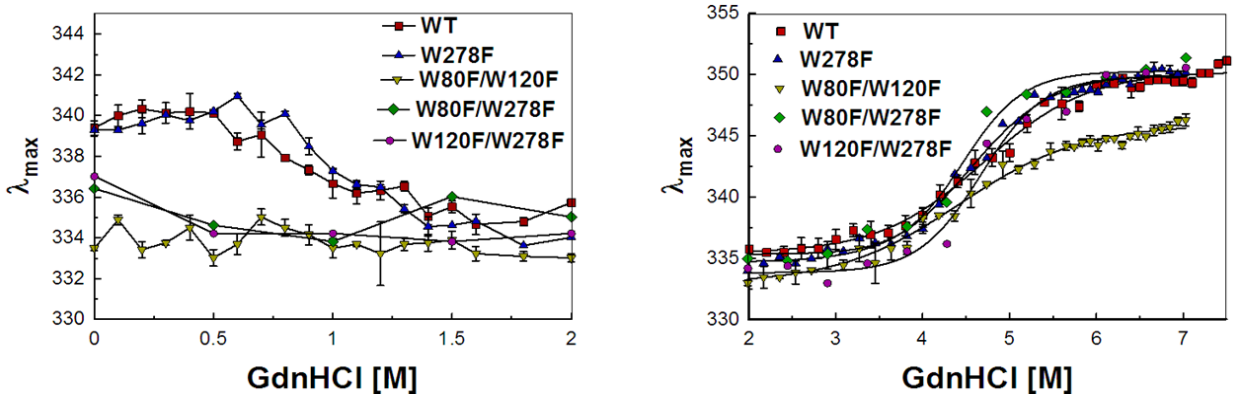


Fig: 5. Equilibrium urea unfolding of WT and W variants of TyrRSapm.

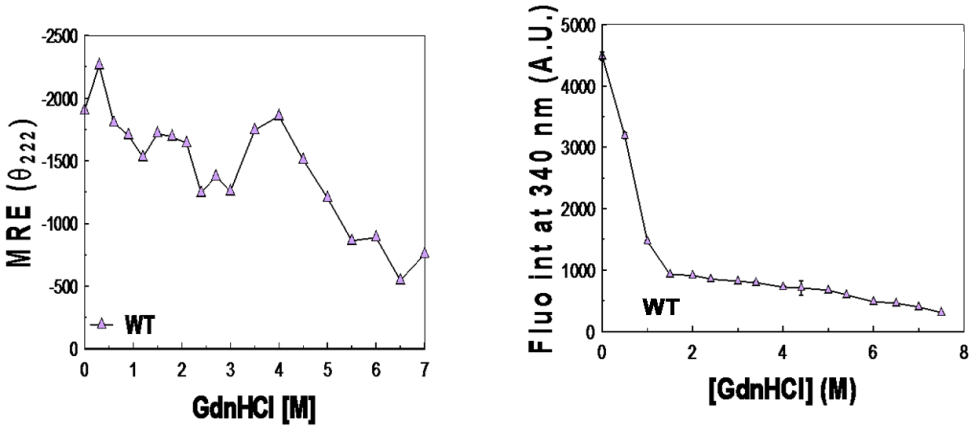
The tryptophan emission maxima data obtained after taking the first derivative of tryptophan emission spectra using HITACHI F-7000 spectrofluorometer with an integration time of 1 s and equipped with a constant-temperature cell holder. The excitation wavelength was 295 nm, and emission was recorded from 310 nm to 450 nm. The excitation and emission slit widths

were both 5 nm. The protein concentration was 4 μM. All the data were repeated three times, and standard deviations were measured.

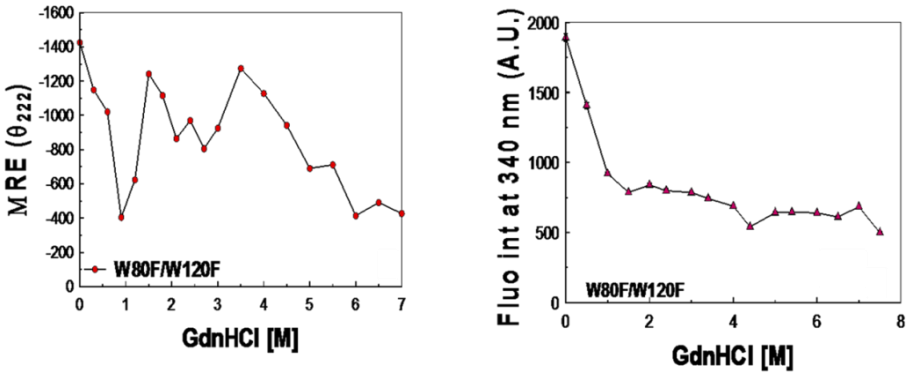
Table: 5. Unfolding parameters of WT and Trp. mutants of TyrRSapm using fluorescenc (from 2-8 M fitting).

	WT	W278F	W80F/W120F	W80F/W278F	W120F/W278F
ΔG	4.67±0.02	5.58±1.22	3.63±1.1	7.35± 0.5	7.45±1.5
M	0.99±0.02	1.16±0.05	0.78±0.01	1.56±0.006	1.51±.008

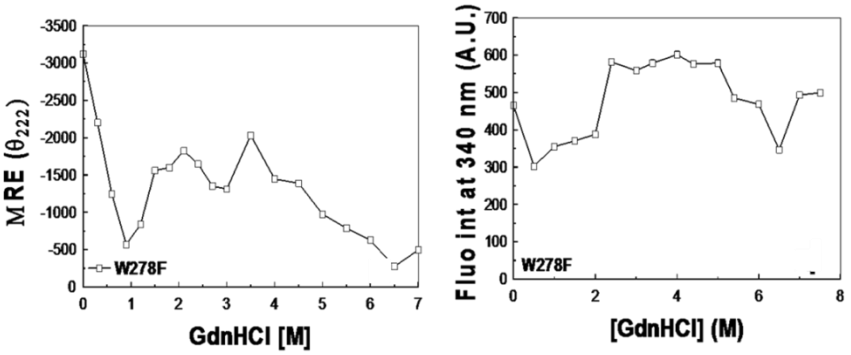
GdnHCl-induced chemical unfolding was performed with increasing concentrations of the denaturant. The tryptophan emission maxima value was recorded as a function of GdnHCl concentration.



A.



B.



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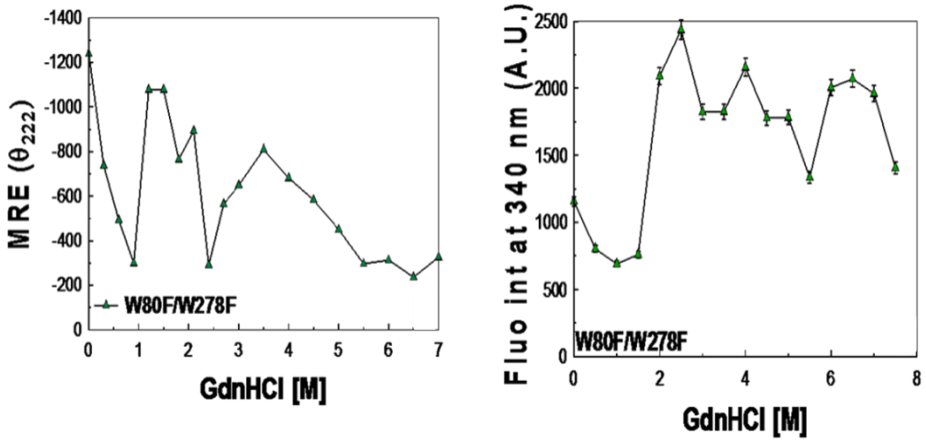
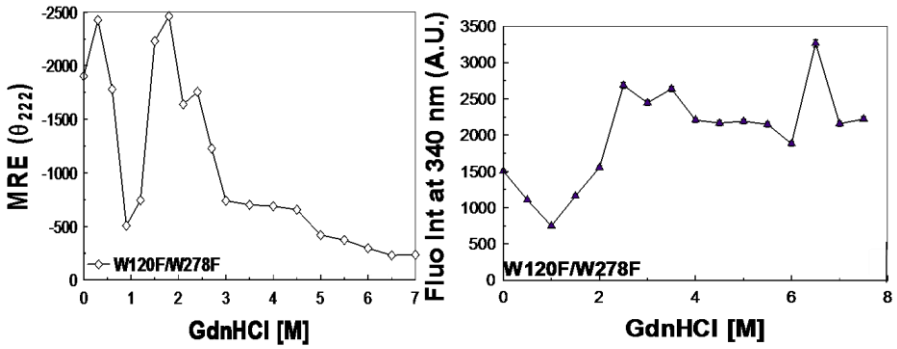


D.



E.

Fig.6. CD (MRE at θ222) and fluorescence intensity of TyrRSapm and its variants at different GdnHCl concentration.

Pannel A to E represents CD and Fluorescence data of WT, W80F/W120F, W278F, W80F/W278F and W120F/W278F respectively. Protein concentrations were 4 µM per monomer and all the sample proteins were in 20 mM Tris-HCl buffer, pH 7.4. The data were

taken from 200 nm to 250 nm in a 1-mm path-length cuvette, with 1 nm of bandwidth and 5 s of averaging time. Five scans were taken and averaged for each sample. Protein-only spectra were obtained by subtracting the CD signal from the corresponding buffer.

Mutants showed similar W accessibility during unfolding

Acrylamide quenching studies of WT and all W mutants (in presence of 0M to 7M GdnHCl conc.) were performed to explore how the accessibility of W residues changed due to gradual structural alterations. Our results demonstrated that the trend of Ksv value change in relation to GdnHCl concentration was almost same for both WT and W mutants of TyrRSapm (Table 6). Hence, during gradual unfolding, accessibility of W residues of mutants was to remain same with that of WT protein.

Table: 6 Ksv(M-1) values of TyrRSapm and its variants at different GdnHCl concentration. Experiment was done using HITACHI F-7000 spectrofluorometer. Protein concentrations were 4µM and buffer composition was 20 mM Tris-HCl, pH 7.4.

	Ksv (M-1)Value				
GdnHCl conc.(M)	**Wt**	**W278F**	**W80F/W120F**	**W80F/W278F**	**W120F/W278F**
0	15.32±0.04	1.89±055	2.7±0.61	2.5±0.062	2.7±0.036
0.3	16.931±0.02	4.5±0.58	3.5±0.54	4.8±0.051	3.3±0.079
0.6	15.65±.003	5.4±0.99	7.5±0.99	8.2±0.02	5.4±17
0.9	11.45±0.02	10±0.38	11.2±093	12.±016	11.6±0.05
1.2	8.25±0.05	5.±0.78	7.81±0.092	11.2±0.06	8.06±05
1.5	5.65±0.02	6.72±42	7.75±0.42	6.34±0.06	5.02±0.09
1.8	4.63±0.02	4.4±0.82	3.5±0.024	2.8±0.07	3.8±0.06
2.1	4.13±0.01	3.12±0.6	4.52±0.06	4.7±0.097	2.3±0.09
2.4	4.17±0.1	3.6±0.05	4.2±0.022	4.36±0.081	2.87±0.03
2.7	3.49±0.22	3.5±0.82	4.09±0.02	4.2±0.066	4.4±0.04
3	3.73±0.48	3.6±0.6	5±0.003	5.2±0.055	4.17±0.09
3.5	5.7±0.13	3.2±0.53	12.1±06	6.6±0.098	5.8±0.07
4	8.±0.53	8±0.97	13.4±0.02	9.7±0.09	7.6±0.05
4.5	9±0.25	10.±0.46	16.13±0.07	11.3±0.09	19.2±0.09
5	11.7±0.21	10.8±0.18	17.09±0.08	13.8±0.06	21.7±0.44
5.5	13.± 0.97	11.3±0.5	18.5±0.08	13.2±0.08	22.14±0.08
6	15.±0.3	12.5±0.23	19.1±0.06	14.3±0.03	24.5±0.04
6.5	16.6±23	17.8±0.91	21.7±0.036	18±.01	25.8±012
7	21.38±0.01	30.6±0.04	26.7±0.03	27.3±0.089	27.1±0.02

Aminoacylation capability varies for mutants

All the structural probes pointed that there were significant structural alteration due to mutations in W residues. Structural perturbation had often associated with loss of enzyme activity [29]. Two of the four mutants namely, W278F and W80F/W120F, had retained the aminoacylation capability (Fig.7), while other two mutants W80F/W278F and W120F/W278F were completely inactive. The activity retained by two mutants may be due to preservation of active site conformation to accommodate the substrate, i.e., substantial loss of structural integrity did not perturb active site. The loss of activity is difficult to explain especially as one of the inactive mutants has similar secondary structure.

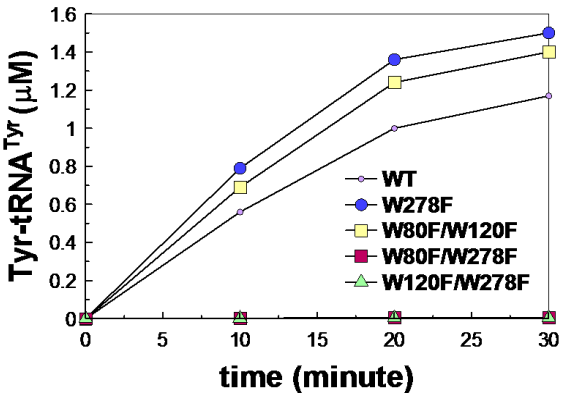


Fig.7. Aminoacylation of TyrRSapm and its variants.

Aminoacylation assay reactions were carried out at 37^0 C for 30 min. Tyrosylation of human tRNATyr transcript was performed in aminoacylation buffer. The amount of radioactivity retained was determined by liquid scintillation counting. The enzyme and tRNA concentration were 20 µM and 3 µM respectively.

Two of the mutants behave differently under the influence of pH change

During cellular translocation, proteins have to pass through pH gradient [30]. To investigate how pH affects the structure of TyrRSapm and its W mutants, we carried out pH induced unfolding. Throughout the pH range the emission maxima remain range bound between 331 nm at pH =1 while 341 nm for pH = 9 (Table 7). The WT showed maximum variation and all of them are largely insensitive to pH. All of them had shown blue shift of emission maxima at lower pH which may be an indication of existence of intermediate form (Table7). The hydrophobic probe, 1,8-anilino napthyl-sulphonate (ANS) binds to exposed hydrophobic sites of proteins and widely use to characterize intermediates such as "Molten Globule" (MG)-state [31], which is larger than the native form of the protein and has an intact secondary structure [31-34]. ANS binding as a function of pH showed that there was a dramatic increase in fluorescence intensity at 482 nm in the range of pH 1 to pH 3, in case of WT-TyrRSapm, W278F and W80F/W120F mutants (Fig.8). The other two inactive mutants namely W80F/W278F and W120F/W278F also showed some increase in that range but are much less pronounced (Fig. 8). This finding indicated that TyrRSapm, W278F and W80F/W120F mutants may unfold through a compact intermediate structure in the given pH values range [24,25]. The anisotropy value (A value) of TyrRSapm and its variants at different pH (Table 8) showed almost no difference as compared to pH =7.4, which indicate that there is no perturbation of dimeric structure at diverse pH range.

Table:7 Change of emission maxima at different pH. Data were recorded with HITACHI F-7000 spectro fluorometer. Protein concentrations were 4 µM.

	EMISSION MAXIMA (ΛMAX)				
pH	**WT TyrRS**	**W278F**	**W80F/W120F**	**W80F/W278F**	**W120F/W278F**
1	333.8±1.3	333±1.19	333.4±1.2	333.8±1.2	330.8±1.1
1.5	334.4±1.1	332.2±1.11	332±1.1	335±1.2	333.8±1.1

2	334.8±1.2	334.6±1.1	335.8±1.2	338.2±1.1	330.8±1.1
2.5	333.8±1.1	332.8±1.1	333±1.2	333.6±1.1	333±1.2
3	334.6±1.1	331.6±1.2	332.6±1.1	332.6±1.2	332±1.1
4	340.2±1.1	332.6±1.1	333.4±1.2	3401.2	334±1.2
5	340.8±1.2	333.8±1.2	336.6±1.1	334±1.3	334.2
7	339±1.2	335.8±1.2	336.4±1.1	335.2±1.1	335.2±1.2
8	338±1.3	333.8±1.1	335.4±1.1	335.2±1.1	336.4±1.1
8.5	341±1.1	335±1.1	336.6±1.2	337±1.2	337.6±1.2
9.5	340.4±1.4	335.4±1.09	335.4±1.1	337.2±1.1	334.8±1.1
10	340±1.3	335.4±1.2	335.6±1.4	337.6±1.1	336.6±1.1
10.5	335±1.1	332.8±1.1	334.2±1.1	337.6±1.1	338.2±1.2
11	335.6±1.4	333.8±1.4	334.4±1.1	335.8±1.2	334.8±1.1
12.5	335.8±1.1	336.2±1.1	335.2±1.1	334.8±1.1	337.6±1.2

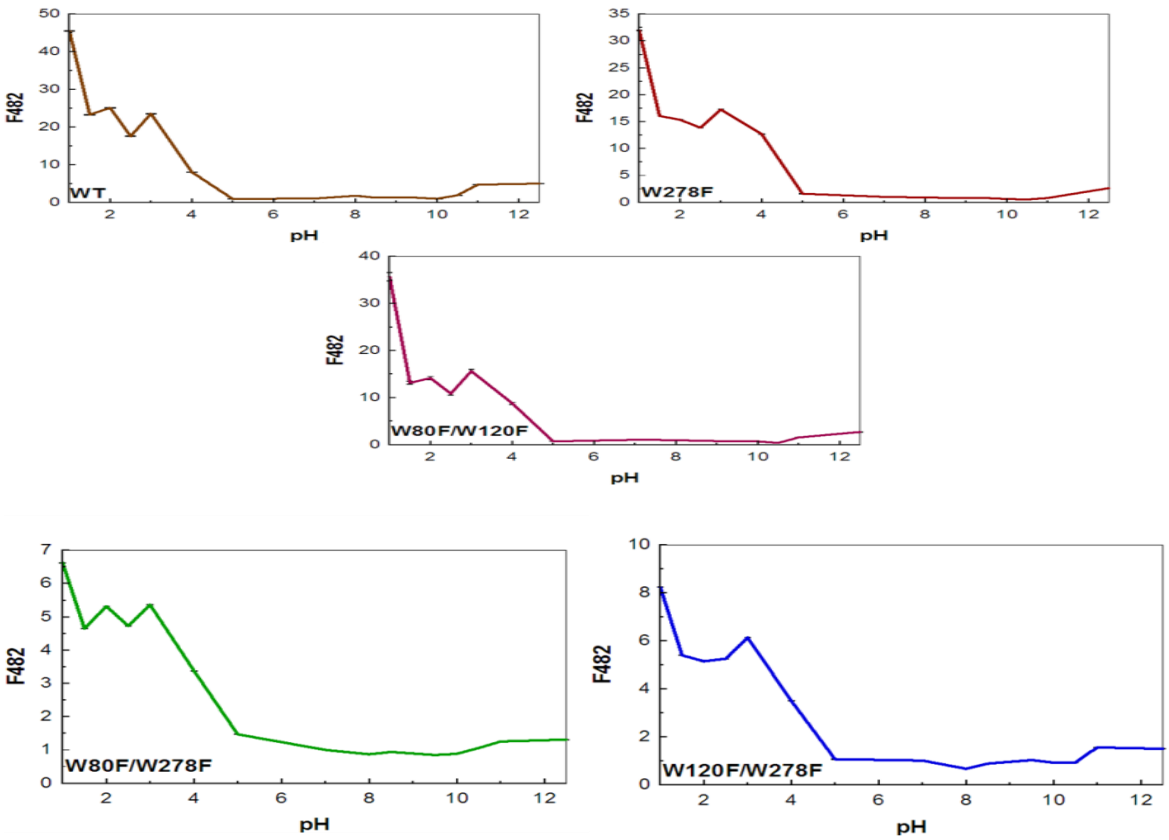


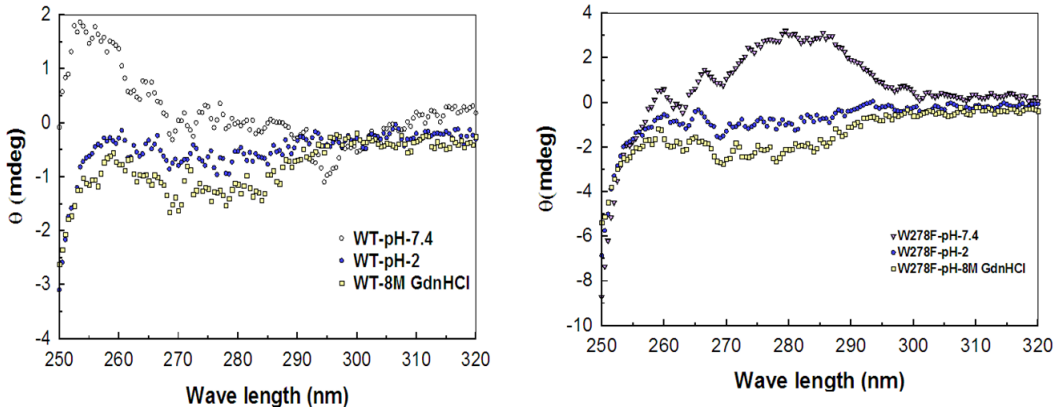
Fig:8. ANS binding during pH induced unfolding of WT and W mutants of TyrRSapm. Protein concentrations were 4 µM per monomer. Each sample was incubated with 30 µM ANS for 30 mins. Samples were excited at 420 nm. Data was recorded with HITACHI F-7000 spectro fluorometer.

Table: 8. Anisotropy value as a function of different pH. Data was recorded with HITACHI F-7000 spectro fluorometer. Samples were excited at 300 nm and emission was set to 350 nm.

	A-VALUE				
pH	**WT**	**W278F**	**W80F/W120F**	**W80F/W278F**	**W120F/W278F**
1	0.137	0.151	0.155	0.253	0.2582
1.5	0.150	0.146	0.156	0.162	0.155
2	0.124	0.142	0.142	0.097	0.144
2.5	0.155	0.154	0.165	0.134	0.137
3	0.139	0.162	0.209	0.141	0.169
4	0.147	0.142	0.148	0.087	0.198
5	0.131	0.197	0.202	0.160	0.249

Two of the mutants exhibited different tertiary interaction as a function of pH

To validate whether the intermediates are MG-state, both far and near UV CD studies at native condition, at lower pH (pH 2) and at completely unfolded condition (8M GdnHCl) were performed (Fig.9). While far UV spectra was unchanged for WT and all the W mutants, at pH 2, near UV CD spectra showed an huge decrease in signal for WT, W278 and W80F/W120F mutants, probing these proteins are passing through a MG-like intermediate conformation [28,29]. For the other two mutants (W80F/W278F and W120F/W278F) the near-UV CD is almost coincidental with 8 M GdnHCl, indicating complete loss of tertiary interaction. Thus these intermediates are definitely not like MG-state.



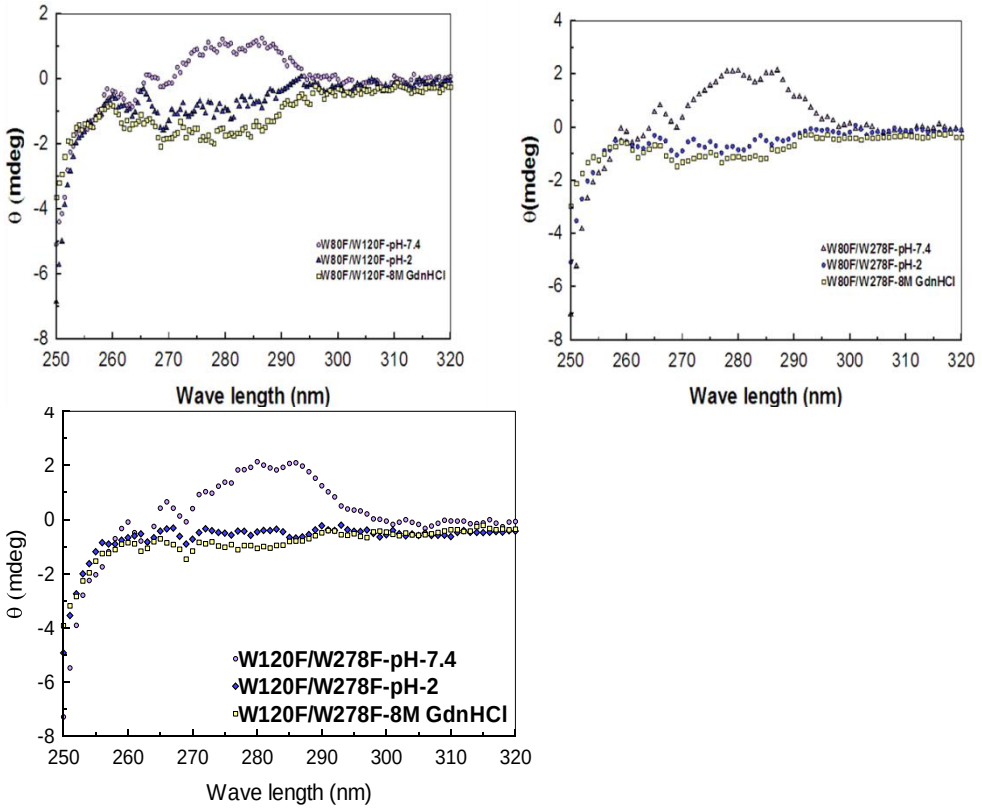


Fig:9. Near UV CD of WT and all Trp. mutants of TyrRSapm at pH 7.4, pH 2 and 8M GdnHCl. For different buffer condition, each sample concentration was 4 µM per monomer.

The protein samples were incubated overnight at 25 °C. Protein-only spectra were obtained by subtracting the CD signal from the corresponding buffer. The protein samples were scanned five times.

Mutations of W residues of TyrRSapm do not perturb the dimeric conformation

The FITC labeled enzyme retained 80% of tRNA aminoacylation activity in comparison to wild-type TyrRSapm. The idea is that at higher concentration the enzyme exists as dimer, thus the tumbling rate is slow, having higher anisotropy value. Upon dilution, it eventually dissociates into monomer with faster tumbling rate, hence lower anisotropy value. Thus plotting anisotropy values as a function of enzyme concentration yields monomer-dimer dissociation constant [17,18]. The monomer-dimer dissociation constant of FITC-labeled TyrRSapm was found to be 0.67 µM.

Results showed that, all the mutants studied here retain dimeric conformation (Fig.10) and dissociates into monomer with similar Kd values like WT TyrRSapm (Table 9). However, it may be noted that continuous dilution of the sample can change the rotational freedom of the probe without affecting the macromolecule, leading to similar changes in anisotropy [11, 35].

To address this issue, two control experiments were done with FITC labeled TyrRSapm and its W mutants. In the 1st control experiment, after gradual dilution of labeled TyrRSapm to nM range, the unlabeled protein was added at once to the sample solution to restore its starting experimental concentration (4 µM). Results showed that anisotropy value was reverted to the

starting value within short period of time. Therefore, it may be suggested that gradual decrease of anisotropy values were due to dimer-monmer dissociation of TyrRSapm, as at higher concentration the protein again retains its dimeric conformation and due to slower rotational freedom, increased anisotropy value was retained.

In a second control experiment the protein concentration was kept constant (4µM) throughout the experiment by adding buffer containing unlabeled protein in each titrating point. Anisotropy values (A-value) of diluted labeled sample were almost unchanged when protein concentration was kept constant. Therefore we can conclude that the decrease of anisotropy values (A-values) upon dilution (Fig 10) was due to dimer to monomer transition of TyrRSapm [36].

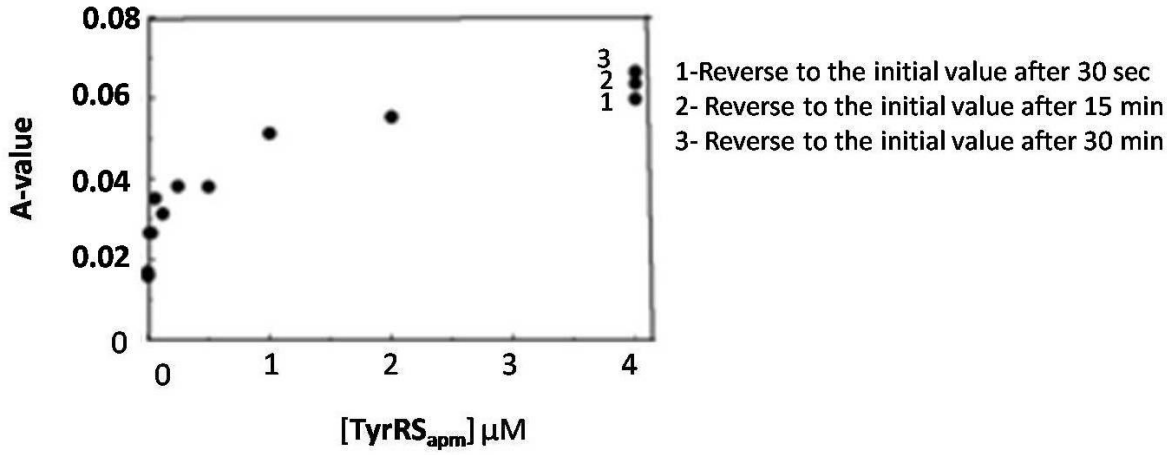


Fig. 10. Steady-state Fluorescence anisotropy of FITC-TyrRSapm showing gradually gaining of initial A-value after addition of unlabeled protein. Unlabeled TyrRSapm (4µM) was added directly to the sample and A value was calculated after 30 sec, 15 min and finally after 30min.

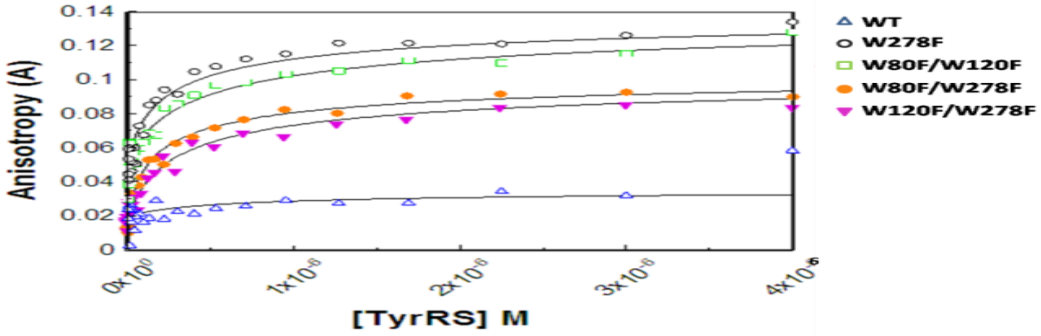


Fig. 11. Steady State Fluorescence Anisotropy of WT and Trp. mutants of FITC-TyrRSapm FITC labeled proteins were excited at 490 nm and emission was recorded at 525 nm. Protein samples were gradually diluted with 20 mM Tris-HCl buffer, pH 7.4. Data was recorded on HITACHI F-7000 spectro fluorometer.

Table: 9. Kd (µM) values of dimer monomer equilibrium of WT and W mutants of TyrRSapm Data was recorded on HITACHI F-7000 spectro fluorometer.

	WT	**W278F**	**W80F/W120F**	**W80F/W278F**	**W120F/W278F**
Kd (µM)	0.6±0.04	0.8±0.008	0.4±0.05	0.5±0.01	0.7±0.06

Protein samples were gradually diluted with 20 mM Tris-HCl buffer, pH 7.4. FITC labeled proteins were excited at 490 nm and emission was recorded at 525 nm.

As the dissociation constant (Kd) values of FITC labeled anisotropy study of W mutants of TyrRSapm are more or less similar to the WT protein, hence it may be concluded thaT mutations of W residues of TyrRSapm do not perturb the dimeric conformation.

Non-canonical orientation has an influence on TyrRSapm dimerization capability

To ensure that dimer-monomer dissociation of TyrRSapm did not alter due to FITC labeling, we carried out sedimentation equilibrium experiment using analytical ultra centrifugation of WT TyrRSapm. This method allows the user to determine the molecular weight of a protein that is independent of its shape. Three different concentrations were analyzed for TyrRSapm (0.2, 0.5, and 2 μM). Global fitting tof he data using monomer-dimer equilibrium model yielded a good fit (having least rmsd value) with dissociation constant (Kd) value of 0.41 μM. This is in good agreement with fluorescence anisotropy study. Figures 12 A, B and C represents the global analysis of the data for TyrRSapm at 0.2, 0.5 and 2 μM concentration respectively, using monomer-dimer association model. The analysis yielded a molecular weight of 84134 Da for TyrRSapm, close to theoretical value calculated based upon amino acid composition (84368 Da including extra amino acids present due to His-tagged extension). Fitting the data using monomer-dimer-tetramer model gave no improvement. This data clearly showed that labeling with FITC did not affect the dimerization capability of TyrRSapm and validated our anisotropy experiment as a reliable approach for dissociation constant (Kd) determination. Furthermore, it indeed showed that non-canonical orientation has an influence on TyrRSapm dimerization capability.

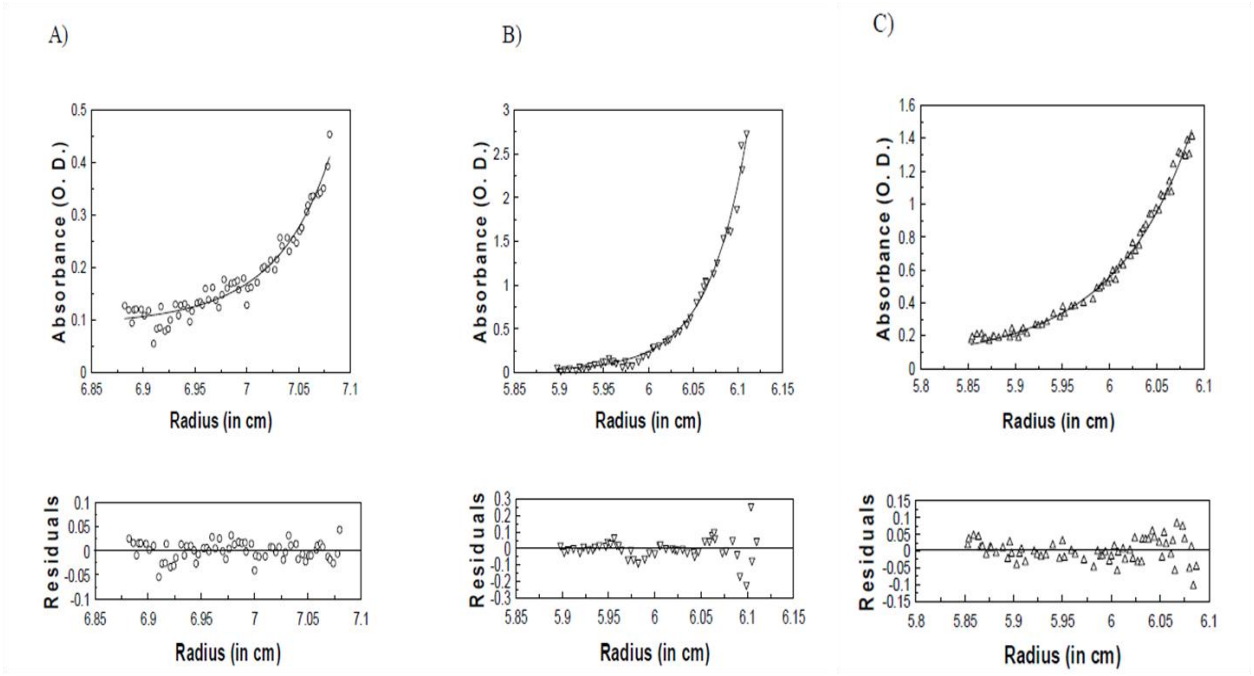


Fig.12. Sedimentation equilibrium analysis of TyrRSapm. A- 0.2μM, B-0.5μM and C 2μM. Data were recorded at 220 nm as a function of radial position at the three loading concentrations of 0.2 μM, 0.5 μM and 2 μM for TyrRSapm. The data were analyzed by global nonlinear least squares fitting using SEDPHAT software (v10. 4, 2012) [12].

TyrRSapm data was fitted in monomer-dimer and monomer-dimer-tetramer model to obtain both molecular weight and dissociation constant. During fitting, both molecular weight and dissociation constant values were allowed to float. Molecular weight (Mr) and dissociation constants were obtained by a model-dependent method with least chi-square and least rmsd value.

Discussion:

The far UV-CD and fluorescence life time measurement clearly indicated a major structural perturbation, however energy transfer between W residues and contribution from W residues in the far-UV region can't be ruled out [16,17]. The energy transfer effect was probably masked by some undefined non-radiative processes. According to crystal structure, W80 and W120 are closely located [2]. W80 and W120 are present at the dimer interface and either of these W residues are surrounded by 3 other W residues (10, 10 and 25 Å, one on the same chain and the other two at the dimeric interface of the other monomer) (Fig. 1) .One would expect inter and intra-chain energy transfer between W80 and W120 leading to quenching of fluorescence in WT and mutants such as W278F. However, the quantum yield revealed a opposite finding, leading to speculation of structural alteration. Acrylamide quenching provided the first hint that the tryptophan microenvironment might have distorted significantly. The more definite proof of structural alteration came from far and near UV-CD. The experimental evidences probed that there is significant change in signal in between 280-300 nm that is largely contributed by W residues [17].

The fluorescence emission maxima did not change much for three of the mutants till 2 M GdnHCl, while WT and W80F/W120F showed blue shifting of emission maxima at this range. For all of the W mutants, the Stern-Volmer plot also largely invariant in this region apparently showing the invariant W microenvironment. Surprisingly, the ellipticity at 222 nm showed large decrease at low concentration of GdnHCl which is very unusual as most of the reports showed either no change or increase in ellipticity at low GdnHCl concentration [38-40]. Two explanation may be proposed, firstly, the contribution of aromatic side-chain CD in far-UV region changed drastically in the presence of low denaturant concentration, secondly, it may be possible that the secondary structure around W residues do not affected at low GdnHCl while the alpha-helix structure is lost in other part of the protein molecule.

One of the unusual features of TyrRSapm is the presence of high number of Cysteine residues (10 per monomer) [2]. While studying accessible cysteine reactivity, we found that there was dramatic variation among the number of reactive cysteine among WT and W mutants. This finding again clearly indicated towards structural perturbation due to W mutation.

The activity of the two mutants like WT protein despite large changes in far UV-CD and near UV-CD spectra under native condition may be explained if the active-site remained unperturbed due to these mutations. This argument is further supported by studying the effect of pH on TyrRS and its mutants, the WT and two active mutants only form "molten-globule" (MG) like intermediate at pH-2.0, while the other two inactive mutants have similar near UV-CD spectra like unfolded proteins, eliminating the possibility of formation of MG-state. It may be concluded that the different folding pathway of inactive mutants may change the

precise orientation of amino acid residues in the active site require to recognize the cognate substrates, leading to inactivity.

Retention of activity of W278F (W80, W120) and W80F/W120F (W278) mutants while inactivity of W80F/W278F (W120) and W120F/W278F (W80) mutants is again a perplexing finding to explain the role of particular W residue and /or residues in function of TyrRSapm. It may be noteworthy to mention here that all W mutants retained their dimeric conformation, however, retention of functional active site conformation of TyrRSapm may require the synergistic effect of W residues [40, 41]. The fact that the activity of the two mutants (W80F/W120F and W278F) were similar to WT enzyme, despite large changes in far UV-CD and near UV-CD spectra under native condition may be explained if the active-site remained unperturbed due to these mutations. This argument is further supported by studying the effect of pH on TyrRS and its mutants, the WT and two active mutants (W80F/W120F and W278F) only form "molten-globule" (MG) like intermediate at pH-2.0, while the other two inactive mutants (/W278F and W120F/W278F) have similar near UV-CD spectra like unfolded proteins, eliminating the possibility of formation of MG-state. It may be concluded that the different folding pathway of inactive mutants may change the precise orientation of amino acid residues in the active site require to recognize the cognate substrates.

The fluorescence anisotropy study with FITC labeled WT TyrRSapm showed the dimer-monomer dissociation constant is in micro molar range (0.67 μM) and this data was verified using analytical ultra centrifugation technique. The AUC data showed that at all concentration tested (0.2, 0.5 and 2 μM); the protein exists both as momomer and dimer in solution, with an estimated dissociation constant of 0.41 μM. This is in sharp contrast to the previously reported dissociation constant of the N-terminal fragment of Bst-TyrRS (84 pM) [1,4] and at least 4-fold weaker to Neurospora crassa mitochondrial TyrRS and human N-terminal catalytic domain of TyrRS, namely TyrRSmini (~0.1 μM) [42]. The higher dissociation constants of later TyrRSs had been attributed to lesser-buried surface area at dimer interface than Bst-TyrRS [2]. Previous study revealed that TyrRSapm has the least buried surface value at dimer interface compare to other TyrRSs (2,360 Å2 for TyrRSapm vs 2,970 to 3,300 Å2 for other TyrRSs) [2]. Considering that buried surface area for TyrRSapm is 20-30% lower compare to other TyrRSs, the weaker monomer-dimer dissociation constant of TyrRSapm (0.41 μM) seems reasonable. The least buried dimeric interface area of TyrRSapm among all known TyrRSs may be accredited to the non-canonical dimeric interface due to the presence of a unique 20-residue long -helix CP1-domain [2], which is known to play central role in dimerization in all TyrRSs. We have previously shown that interfacial W residues may play some crucial role in structure and function of the enzyme. However, the dissociation constants of W mutants clearly demonstrated that mutants have almost similar affinities for their sub units. Thus W residues present in the dimeric interface don't have any influence on dimerization of TyrRSapm.

Conclutions:

The sequence alignment of TyrRSapm with TyrRSs of other sources did not reveal any conserved W residue [2], however we found that two of them are crucial for tRNA binding as W80F/W28F and W120F/W278F are in active in aminoacylation and all of them showed

structural perturbation. Mutation of non conserved residues leading to structural perturbation as well as functional loss was very rare. However, like other unique features of TyrRSapm namely noncanonical dimer orientation [2], probable possible 4th domain of life [6]; our study enriches the list by marking all of three W residues as essential non-conserved residues.

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