Uncovering of a Short Internal Peptide Activates a tRNA Synthetase Procytokine

Aminoacyl-tRNA synthetases are ancient enzymes that catalyze the first step of protein synthesis by attaching amino acids onto their cognate tRNAs (1). In addition, in higher organisms, aminoacyl-tRNA synthetases gained a variety of expanded functions in cell signaling pathways ranging from vascular to inflammatory pathways, among others (2–6). These expansions, often implemented with secreted forms, developed in evolution through the incorporation of novel domains and sequence motifs that bear no impact on the protein synthesis function. That these additions were added at precise time points in evolution and are tightly regulated suggests that they played a role in the development of higher organisms (7–9).

Human tyrosyl-tRNA synthetase (TyrRS)2 is a 528-amino acid procytokine composed of two domains connected by a long linker that spans ~20 amino acids (Fig. 1A). The 364-amino acid N-terminal part (mini-TyrRS) is the catalytic aminoacylation domain and is active without the C-terminal domain (CTD) (10, 11). At the evolutionary stage of insects, an ELR tripeptide was incorporated into the mini-TyrRS domain and has been retained in higher organisms. The ELR motif is the recognition element within CXC chemokines such as interleukin-8, which mediates the innate immune response as well as angiogenesis (12, 13). Simultaneously, the CTD, which is a structural and functional homolog of human endothelial monocyte-activating polypeptide II (EMAP II), was appended to TyrRS. Previous work established that mini-TyrRS and the CTD have distinct cytokine activities associated with hematological and inflammatory pathways, whereas full-length TyrRS is inactive as a cytokine (14, 15). Thus, the novel activities are observed only when the two domains of secreted TyrRS are separated by natural proteolysis at the interdomain linker.

The crystal structures of mini-TyrRS and the CTD have separately been determined (16, 17). In the mini-TyrRS domain, the ELR motif is masked by the terminal α-helix 14, which is tethered by a hydrogen bond between the hydroxyl of the Tyr-341 side chain and the main chain carbonyl of Gly-46 (Fig. 1B). Ablation of this hydrogen bond by a Y341A substitution confers IL-8-like cytokine activity on the full-length TyrRS without proteolysis at the interdomain linker (18). These results stimulated our interest in understanding the structural relationships of TyrRS, Y341A TyrRS, and mini-TyrRS in the light of the cytokine activity.

Our goal was to figure out whether the mini-TyrRS cytokine activity could be explained by specific unmasking of the critical ELR motif or whether more dramatic changes occur that result in additional surfaces being perturbed and provide an important context for ELR signaling. In previous work, we used mutational analysis to establish that the cytokine activity was dependent on the ELR motif (11, 14, 18–20) and functioned through CXCR1 and -2 receptors (21). Further, we used a low resolution protease mapping procedure to show that the ELR-dependent activity was associated with a specific conformational change (18). However, we could not definitively prove (with the methods used) that this conformational change affected the exposure of ELR. To address this question, we took advantage of Y341A TyrRS and surmised that, at the least, the disposition of the ELR motif must be altered by the Y341A mutation. As it turned out, TyrRS and Y341A TyrRS were both recalcitrant to extensive efforts to obtain crystals, possibly
followed by simultaneous quench and proteolysis (25). Reactions were quenched by 1:1 (v/v) addition of protease type XIII solution in 1% formic acid to reduce the pH to ~2.3 and to initiate the 2 min of proteolysis. The digested peptide fragments were separated by a fast LC gradient through a ProZAP C18 column (Grace Davison, 1.5 µm, 500 Å, 2.1 × 10 mm) to minimize back exchange. A post-column splitter reduced the LC eluent flow rate to ~400–500 nL/min for efficient microelectrospray ionization. Microelectrosprayed HDX samples were directed to a custom-built hybrid linear trap quadrupole 14.5-tesla FT-ICR mass spectrometer (Thermo Fisher) (26). The total data acquisition period for each sample was 6 min. Each experiment was performed in triplicate. Data were analyzed by an in-house analysis package (27). Time-course deuterium incorporation levels were generated by a maximum entropy method fitting method (28).

SAXS—Just prior to SAXS experiments, protein samples were purified by gel filtration to remove aggregates in a buffer containing 100 mM NaCl, 20 mM HEPES, pH 7.5, 2 mM β-mercaptoethanol, and 3% (v/v) glycerol. SAXS data were collected at beamline 4-2 at Stanford Synchrotron Radiation Lightsource (SSRL). Measurements on protein samples at 2, 3, 5, and 10 mg/ml at 10 °C were collected on a MarCCD 165 detector. No systematic differences were detected across concentrations, indicating the absence of interparticle interference. Data were processed by SasTool (MarParse) and PRIMUS (29). The forward scattering I(0) and the radius of gyration $R_g$ were estimated from the Guinier approximation under that at very small angles ($\langle s < 1.3/R_g \rangle$); the intensity is represented as $I(s) = I(0)\exp(-sR_g^2/3)$. Maximum particle dimensions $D_{max}$ were computed by use of the indirect transform package GNOM, which also gives the distance distribution function $P(r)$. Ab initio free atom modeling was performed with GASBOR (30). 35 independent simulations were carried out for TyrRS and Y341A TyrRS using 2-fold symmetry. Superposition, averaging, and filtering with DAMAVER (31) generated the final reconstructions, and envelopes were constructed using the Situs software (32).

RESULTS

We scanned the solvent-accessible regions of TyrRS and Y341A TyrRS by HDX-MS. Interestingly, both full-length proteins showed no differences in deuterium uptake in nearly all regions (Fig. 2A). In particular, the HDX-MS profiles of the 172-amino acid CTD of Y341A TyrRS were similar to that of TyrRS within −10% to +10% difference. Most of the HDX-MS profiles of the mini-TyrRS portions were similar, although the additional changes that did occur suggest a mild relaxation, or loosening, of Y341A TyrRS. Although inclusion of CTD was shown to stabilize the dimer interaction of TyrRS that is mediated through the mini-TyrRS portion of the protein (18), the interaction between mini-TyrRS and the CTD is observed neither computationally (33) nor by a direct test in solution; e.g. the two domains do not associate and assemble into a stable complex in solution and presumably roll around freely when tethered by the interdomain linker (supplemental Fig. S1), further explaining the equivalent CTD HDX-MS profiles. In contrast, a hexapeptide spanning the ELR motif at positions 90–95 in the

FIGURE 1. Structural organization of dimeric human TyrRS. A, domain organization of human TyrRS with the Rossmann fold and the anticodon recognition subdomain of mini-TyrRS in blue and green, respectively, joined through a linker (black) to the CTD in red. The ELR motif and the Tyr-341 residue are in orange and red, respectively. B, overall structural model of TyrRS with one subunit of mini-TyrRS (Protein Data Bank (PDB) 1N3L) and the CTD (PDB 1NGQ) colored as in Fig. 1A. Dashed lines indicate the unmodeled interdomain linker. The magnified view shows the hydrogen bond interaction between Tyr-341 and Gly-46 at a distance of 2.3 Å.

because of the flexibility of the long interdomain linker. In addition, because both proteins are too large for straightforward assignment of peak assignments and NOE signals by NMR, we turned instead to methods that could survey the entire protein and give specific information about the disposition of the ELR motif. For this purpose, we used quantitative hydrogen/deuterium exchange of amide protons by mass spectrometry (HDX-MS) (22) and small-angle x-ray scattering (SAXS) solution methods for our structural analysis.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—All variants of TyrRS were cloned in the pET-20b vector (Novagen) using NdeI and XhoI restriction cut sites. Full-length TyrRS is composed of residues 1–528 and mini-TyrRS spans residues 1–364. Proteins were expressed in Escherichia coli BL21(DE3) cells (Invitrogen) by isopropyl 1-thio-galactopyranoside induction (0.2 mM) at room temperature for 16 h. Cell pellets were resuspended in a buffer containing 500 mM NaCl, 20 mM Tris, pH 8, and 2 mM β-mercaptoethanol, and cells were lysed through an M-110L pneumatic Microfluidizer (Microfluidics). The lysate was clarified by centrifugation, and proteins were purified by nickel-nitrilotriacetic acid (Qiagen) and anion exchange chromatography (GE Healthcare). Samples were buffer-exchanged into 20 mM NaCl, 10 mM HEPES, pH 7.5, and 2 mM β-mercaptoethanol and concentrated. Aliquots were flash-frozen in liquid nitrogen and stored at −80 °C.

HDX Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR MS)—HDX FT-ICR MS methods have been described previously (23, 24). Briefly, 5 µl of sample (30 µM) was mixed with 45 µl of buffer containing 10 mM HEPES, pH 7.5, 20 mM NaCl in D$_2$O to initiate each HDX reaction. HDX incubation periods were 0.5, 1, 2, 4, 8, 15, 30, 60, 120, and 240 min.
sequence showed 64% increased HDX, consistent with a specific unmasking of ELR (Fig. 2B). Mapping the increased deuterium uptake differences onto the structure of the mini-TyrRS showed that the main relaxation of the structure is localized to regions immediately surrounding the ELR motif (Fig. 2C). In addition, we investigated the HDX-MS profiles for mini-TyrRS and compared them with the equivalent portion of native TyrRS. This experiment was meant to test from a structural perspective whether the ELR-associated activity of mini-TyrRS mimicked the changes of Y341A TyrRS. This is of particular interest because of the dramatically different ways in which cytokine activity is activated: by removal of an entire CTD in the case of mini-TyrRS or solely by a single point mutation as in Y341A TyrRS. Remarkably, the HDX profile of mini-TyrRS closely matched that of Y341A TyrRS, with the hexapeptide encompassing the ELR motif showing the greatest exchange (Fig. 2A). Thus, the functional equivalence of Y341A TyrRS and mini-TyrRS appears to be entirely due to the similar way in which they each achieve, by different mechanisms, a pinpointed exposure of the ELR motif.

We reinvestigated the shapes of TyrRS and Y341A TyrRS in solution by calculating molecular envelopes using SAXS profiles. Previous work had only described the pairwise distribution function, but no shapes were calculated (18) because the data were influenced by aggregation and therefore not ideal. At first glance, the experimental scattering curves as well as the pairwise distribution functions of each protein look identical (supplemental Fig. S2). A total of 35 individual reconstructions were calculated and merged to generate envelopes of TyrRS and Y341A TyrRS. Both molecules are organized with mini-TyrRS in the center and two CTDs protruding from opposite sides to form puckered S-shaped molecules (Fig. 3A). However, a subtle difference was observed between TyrRS and Y341A TyrRS at the interdomain linker region, where the CTDs point in opposite directions and thereby alter the respective pucker of each molecule. Strikingly, the ELR area is masked by the CTD in FIGURE 2. Comparisons of HDX-MS kinetics of Y341A TyrRS and mini-TyrRS versus full-length wild-type TyrRS. A, sequence representation of differences in deuterium incorporation between Y341A TyrRS and mini-TyrRS against TyrRS (92 and 85% sequence coverage, respectively). The values were calculated by taking the average of the relative difference of each exchange time point. B, deuterium uptake time-course curves for the ELR-containing peptide, Leu-90 – Ser-95, of Y341A TyrRS and TyrRS. Error bars indicate S.D. C and D, deuterium uptake differences of Y341A TyrRS and mini-TyrRS versus TyrRS, respectively, mapped onto a subunit of mini-TyrRS (PDB 1N3L), in which the colors reflect those from the sequence representation, with (for simplicity) the few sequences not covered also colored in gray (the same as those that have no significant change in deuterium uptake). The ELR motif and the Tyr-341 residue are labeled and are represented as sticks.
TyrRS, but is exposed in Y341A TyrRS (Fig. 3B). Although the handedness of SAXS envelopes cannot be resolved (34), the translocation of the CTDs in these models corroborates well with the localized HDX-MS uptake values at the ELR motif.

Although mini-TyrRS contains the Tyr-341 gatekeeper residue, it possesses ELR-dependent cytokine activity (11). An explanation is that the temperature factors of the helices that contain the ELR motif and Tyr-341 residue are relatively high in the solved mini-TyrRS crystal structure (supplemental Fig. S3). Thus, these regions are disposed to greater conformational flexibility by which the ELR motif is revealed as observed by HDX-MS (Fig. 2D). Despite having high temperature factors in these helices, because TyrRS is inactive as a cytokine, it is likely that the interdomain linker and the CTD occlude the ELR motif, as observed by the SAXS envelopes.

DISCUSSION

Our results demonstrate a remarkable specificity and convergence to the location of the structural change caused by the designed Y341A mutation and by the natural proteolytic product of TyrRS; using solution techniques, we demonstrated that the structural effect on the disposition of the ELR motif of Y341A TyrRS closely mimics that caused by removal of the CTD from TyrRS. Interestingly, mini-TyrRS is active in aminoacylation and can even sustain cell growth as the sole source of tyrosine (10). Recent work showed that the ELR-associated activity of mini-TyrRS is also dependent on its dimeric structure (21).

During the long evolutionary history of this protein, neither the introduction of the ELR motif nor the CTD adversely affected the viability of aminoacylation. However, the two apparently coincident events, introduction of the ELR and the CTD, appear to have been designed so that the CTD would regulate the new activity brought in by the ELR in the N-terminal part of the protein. This change in TyrRS was done in a way that did not disturb its capacity for aminoacylation, and that constraint may have necessitated that the architecture of the protein be especially amenable to accommodating a pinpointed exposure of ELR.

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