Dissociating Quaternary Structure Regulates Cell-signaling Functions of a Secreted Human tRNA Synthetase*\(^{5}\)

My-Nuong Vo, Xiang-Lei Yang\(^1\), and Paul Schimmel\(^2\)

From the Skaggs Institute for Chemical Biology and Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037

Aminoacyl-tRNA synthetases are a family of ancient enzymes essential for decoding genetic information in translation (1). Surprisingly, many tRNA synthetases and synthetase-binding proteins have been expropriated for alternative functions in biological pathways not directly connected to translation. These include roles in angiogenesis (2–5), the inflammatory response (6–8), and immunomodulation (9), involving interactions with cell surface receptors, mRNAs, and nuclear partners (10), and are conferred or regulated by novel domains and sequence motifs added progressively during the evolution of eukaryotes (11). Mechanisms for activation of these expanded functions include post-translational modification, e.g. phosphorylation (7, 10), alternative splicing, or natural proteolysis (4, 5, 12). The variety of disease-associations with tRNA synthetases that have been annotated (13–15), including the identification of casual mutations (16, 17), is thought to reflect, at least in part, the connections of tRNA synthetases to these alternative functions (11).

Tyrosyl-tRNA synthetase (TyrRS)\(^3\) is a homodimer through-out evolution. Although the catalytic site for attachment of activated tyrosine to the 3’-end of tRNA\(^{\text{TyT}}\) is entirely contained within the monomer unit, the dimeric state is required for amino acid activation (and therefore for aminoacylation activity) (18, 19). This requirement is due partially to a conformational change across the dimer interface (20). The overall design of TyrRS is different as the tree of life is ascended (21). For example, human TyrRS has a C-terminal domain not found in orthologs of lower eukaryotes, archaeabacteria, or prokaryotes (see Fig. 1) (11, 22). Previous work showed that although native human TyrRS is inactive as a cytokine, under inflammatory conditions, it can be secreted and split by leukocyte elastase into two fragments having distinct cytokine activities (4). The C-terminal domain, which is the structural homolog of the mature form of human endothelial monocyte-activating polypeptide II (EMAP II), has potent leukocyte and monocyte chemotaxis activity and stimulates production of myeloperoxidase, tumor necrosis factor-\(\alpha\), and tissue factor (4).

On the other hand, the N-terminal catalytic domain TyrRS\(^{\text{Mini}}\) functions in part as an interleukin-8 (IL-8)-like cytokine that stimulates migration of polymorphonuclear (PMN) cells through a mechanism that requires an ELR tripeptide motif like that found in CXC cytokines (4, 5, 23). This tripeptide motif is masked in TyrRS but is exposed when the C-domain is removed to release TyrRS\(^{\text{Mini}}\). The stimulating effect of TyrRS\(^{\text{Mini}}\) on PMN cell migration has a bell-shaped concentration dependence (4, 24, 25), similar to that seen with IL-8 family members. In the case of IL-8, increasing concentrations are associated with desensitization through internalization of its CXCRI/2 receptors (26–28).

In exploratory experiments (see below), we found that although receptor internalization could be demonstrated for CXCRI- or CXCR2-expressing HEK 293 cells treated with IL-8, no such internalization was seen when the same cells were treated with TyrRS\(^{\text{Mini}}\). To understand this observation, we considered the possibility that TyrRS\(^{\text{Mini}}\) was a monomer at concentrations (10 nM) where it acted as an agonist for PMN
cell migration. Considering that the $K_d$ for the monomer-dimer equilibrium of *Neurospora crassa* mitochondrial TyrRS (which is orthologous to TyrRS$^{\text{Mini}}$) is ~100 nm (29) and considering the smaller dimer interface seen in our high-resolution (1.18 Å) structure of human TyrRS$^{\text{Mini}}$ (22) as compared with that of *N. crassa* mitochondrial TyrRS (30), we inferred that the $K_d$ of TyrRS$^{\text{Mini}}$ would be ~100 nm. These considerations led us to hypothesize that monomeric TyrRS$^{\text{Mini}}$ is the active cytokine. However, this hypothesis alone does not explain the bell-shaped concentration dependence, and for that reason, we were also interested in exploring the role of the dimer that is formed at higher concentrations. For this purpose, we set out to determine the $K_d$ for the monomer-dimer equilibrium and also to investigate the activities of rationally designed non-associating monomers and non-dissociating dimers. The results of these investigations support the view that the monomer-dimer equilibrium is a critical regulator of the cytokine function of human TyrRS.

**EXPERIMENTAL PROCEDURES**

Details of experimental protocols are given in the supplemental material. The receptor internalization assay was done with HEK 293 cells that stably express CXCR1 or CXCR2 (a gift from Dr. Adit Ben-Baruch at Tel Aviv University). The plasmid encoding wild-type (WT) human TyrRS$^{\text{Mini}}$ was been cloned previously (4) and was used to generate TyrRS$^{\text{Mini}}$ variants by site-directed mutagenesis using the QuikChange$^\text{TM}$ mutagenesis kit from Stratagene (La Jolla, CA). Circular dichroism (CD) spectra were obtained with an Aviv model 400 CD spectrometer (Aviv Biomedical, Inc. Lakewood, NJ). Analytical gel chromatography was done by injecting each protein sample (200 µL of 10 µM) onto a Superdex 200 chromatography column (GE Healthcare, 10/300GL) in PBS containing 5 mM β-mercaptoethanol. Amino acid activation assays were performed at 25 °C as described previously (31), with some modifications. Receptor binding assays were done with CXCR1- and CXCR2-transfected HEK 293 cells that were incubated with purified His$_6$-tagged WT TyrRS$^{\text{Mini}}$ or TyrRS$^{\text{Mini-Mono}}$. The Transwell cell migration assay was done with human PMN cells that were prepared from heparin-treated whole blood obtained from healthy volunteers using the RosetteSep human granulocyte enrichment kit (StemCell Technologies, Vancouver, BC, Canada).

**RESULTS**

*Testing for Receptor Internalization*—Several laboratories provided evidence that internalization of CXCR1 and CXCR2 receptors upon treatment of CXCR1- or CXCR2-expressing HEK 293 cells with IL-8 accounts for the bell-shaped response of the migration of these cells to the concentration of exogenously added IL-8 (26, 27). We explored receptor internalization by using the same HEK 293 cells transfected with genes expressing either CXCR1 or CXCR2. Using FACS analysis with both the CXCR1 and the CXCR2 cell lines, we observed a strong reduction of the fluorescent signal from fluorescein-labeled α-CXCR1 or α-CXCR2 antibodies bound to cells treated with IL-8 (Fig. 1, c and d). This reduction corresponds to the change associated with receptor internalization. In contrast, treatment of the same cells with TyrRS$^{\text{Mini}}$ induced no change, suggesting that TyrRS$^{\text{Mini}}$ does not promote receptor internalization.

*Rationally Designed Trapped Monomeric and Dimeric TyrRS$^{\text{Mini}}$*—The Rossmann fold of all class I tRNA synthetases is split by an insertion known as connective polypeptide 1 (CP1) (32). This insertion makes important contacts for formation of the dimer interface of human TyrRS$^{\text{Mini}}$. In particular, our three-dimensional structure showed that Pro-159, Leu-160, and Leu-161 in CP1 are involved in backbone hydrogen-bonding interactions that stabilize the two subunits (22) (Fig. 1b). Previously, monomeric TyrRS$^{\text{Mini}}$ (designated here as TyrRS$^{\text{Mini-Mono}}$) was generated by a Δ159–161 deletion, which resulted in a stable recombinant protein that could be
expressed and purified in *Escherichia coli* (24). To generate a non-dissociating dimer, a disulfide trap strategy was employed. This strategy has been successfully used to create a non-dissociating IL-8 dimer (33–35). With this method, an exposed cysteine is introduced at the dimer interface of each individual monomer so that the -SH groups are spatially proximal in the dimer. Upon oxidation, a single disulfide link can form across the dimer interface.

Inspection of the structure of TyrRSMini suggested that the Thr-130 side chain OHs of each subunit were separated by only 3.5 Å and therefore could be tried as sites to introduce cysteine replacements (Fig. 1b). Recombinant T130C TyrRSMini was created and expressed in and purified from *E. coli*. Subsequent I$_2$ oxidation led to the formation of a disulfide bond between the two subunits to give a stable dimer designated as TyrRSMini-Dimer (supplemental Fig. 1a).

As expected (18, 19, 21), TyrRSMini-Mono was inactive for aminoacylation. TyrRSMini-Dimer was also inactive. However, the uncross-linked T130C TyrRSMini was fully active, suggesting that flexibility of the dimer interface was needed for catalysis (supplemental Fig. 1b). Far-UV CD measurements (to monitor secondary structure) of TyrRSMini-Mono, TyrRSMini-Dimer, and TyrRSMini$^{\text{C130S}}$ were similar (supplemental Fig. 1c), and the thermal melting profile showed no significant difference in the thermal stability of TyrRSMini-Mono as compared with TyrRSMini$^{\text{C130S}}$ (supplemental Fig. 1d). Interestingly, TyrRSMini-Dimer had a melting curve with the same shape as those of TyrRSMini-Mono as compared with TyrRSMini$^{\text{C130S}}$ but shifted 7.5 °C to higher temperatures, as expected for the extra stabilization provided by the covalent intersubunit linkage (supplemental Fig. 1d). These results collectively suggested that all three proteins were properly folded.

**Investigation of Monomer-Dimer Equilibrium by Gel Filtration**—Analytical gel filtration chromatography, at a concentration of 10 μM, showed that as expected, TyrRSMini and TyrRSMini-Dimer eluted as dimers, whereas TyrRSMini-Mono migrated mostly as a monomer (Fig. 2a). By varying the protein concentration, the apparent dissociation constant for the dimer-monomer equilibrium of native TyrRSMini was estimated as a $K_d$ value of $100\text{nM}$ (Fig. 2b). In contrast, the apparent dissociation constant for TyrRSMini-Mono$^{\text{C130S}}$ was estimated as $100\text{μM}$ (Fig. 2c), $1000\text{-fold}$ higher than that of TyrRSMini.

**TyrRSMini-Mono and TyrRSMini-Dimer Bind to CXCR1 and CXCR2 Receptors**—CXCR1 was initially identified as a receptor for TyrRSMini in PMN cells (4). Subsequent work established CXCR2 as a second receptor. For this purpose, we used confocal microscopy with HeLa cells transiently expressing CXCR1 or CXCR2. We chose HeLa cells because their morphology made them particularly amenable to visualizations by confocal microscopy. We generated HeLa cell lines transiently expressing CXCR1 or CXCR2 receptors. Twenty-four hours after cells were transfected, they were treated with purified His$_6$-TyrRSMini for 1 h at 4 °C. After treatment, cells were washed twice with PBS and then fixed for immunofluorescence staining using anti-His$_6$- and anti-V5-antibodies for TyrRSMini and CXCR1/2 receptors, respectively. Fig. 2d shows that cells expressing CXCR1 or CXCR2 (red staining) have a much higher density of binding TyrRSMini$^{\text{C130S}}$ (green staining) than do parental HeLa cells. In further work, we investigated stably transfected HEK 293 cells that were stably transfected with each receptor. (The morphology on plates of HEK 293 cells makes them less amenable to visualization techniques using confocal microscopy.) HEK 293/CXCR1 or HEK 293/CXCR2 cells were incubated with 100 nM of purified His$_6$-tagged TyrRSMini or TyrRSMini-Mono. The binding of exogenously added TyrRSS was detected with α-His$_6$ antibodies. Fig. 2e shows that after treatment with 100 nM of either TyrRSMini or TyrRSMini-Mono and using Western blots (with α-His$_6$ antibodies) of PAGE gels that resolved proteins bound to cells expressing either CXCR1 or CXCR2, TyrRSMini and TyrRSMini-Mono each bound to cells expressing either receptor. (Note that in these experiments, the input of TyrRSMini is less than that of TyrRSMini-Mono. After normalization to the input, the binding of TyrRSMini and TyrRSMini-Mono was closely similar.) In contrast, no binding was observed to the non-receptor-expressing parental HEK 293 cell line (Fig. 2e).

The α-His$_6$ antibodies used in these studies only weakly reacted with TyrRSMini-Dimer, and for that reason, reliable data at 100 nM were not obtained and are omitted. As a way to study binding of TyrRSMini-Dimer, we investigated whether TyrRSMini-Dimer can compete with TyrRSMini for binding to the two receptors. We found that increasing concentrations (above 100 nM) of TyrRSMini-Dimer displaced binding of TyrRSMini (Fig. 2f).

**Monomer Is an Agonist and Dimer Is an Antagonist of Induced PMN Cell Migration**—TyrRSMini has potent activity for inducing the migration of human PMN leukocytes (4, 24, 25). We investigated the PMN cell migration activity of TyrRSMini-Mono and TyrRSMini-Dimer. At low concentrations, TyrRSMini-Mono and TyrRSMini stimulated migration to roughly the same degree (compare Fig. 3a at 1 and at 10 nM). (Because the apparent $K_d$ for the dimer-monomer equilibrium of TyrRSMini is $100\text{nM}$, TyrRSMini is predominantly a monomer at low nanomolar concentrations.) At higher concentrations, TyrRSMini showed the characteristic bell-shaped dependence of migration on concentration. In contrast, TyrRSMini-Mono, at concentrations up to 1 μM, did not show a large diminution in stimulation of cell migration. In contrast, no PMN cell migration activity was observed when TyrRSMini-Dimer was used. Collectively, these results showed that the monomer of TyrRSMini is the active and the dimer is the inactive ligand for PMN cell migration.

The decrease in cell migration activity of TyrRSMini as the concentration was raised coincides with the concomitant increase in the dimer-to-monomer ratio of TyrRSMini. Because TyrRSMini-Dimer is not active for stimulation of cell migration and because it competes with TyrRSMini for binding to CXCR1 or CXCR2, we speculated that the dimer is a non-functional receptor-binding form that, at high concentrations, blocks binding of monomeric TyrRSMini. Because it can block monomeric TyrRSMini, the cell migration activity is reduced and, at the limit of high concentrations, there should be little or no migration. To test this hypothesis, we performed competitive cell migration assays in which we pretreated PMN cells with progressive increases in the concentration of TyrRSMini-Mono or TyrRSMini-Dimer. Consistent with the bell-shaped response of PMN cell migration to the concentration of TyrRSMini, but...
not TyrRS\textsuperscript{Mini-Mono}, inhibited cell migration in a dose-dependent manner (Fig. 3b).

Lastly, the lack of receptor internalization seen with TyrRS\textsuperscript{Mini} was also seen with TyrRS\textsuperscript{Mini-Mono} and TyrRS\textsuperscript{Mini-Dimer} (Fig. 1, c and d). Thus, other than the difference of agonist versus antagonist activity, the rationally designed TyrRS\textsuperscript{Mini-Mono} and TyrRS\textsuperscript{Mini-Dimer} behave like TyrRS\textsuperscript{Mini}.

**DISCUSSION**

Fig. 3c summarizes our results showing that dimer dissociation switches secreted TyrRS\textsuperscript{Mini} between two opposing activities for cell signaling. Thus, the present work supports an emerging theme of a role for alternative quaternary structures in the novel functions of human tRNA synthetases. For example, mutations in homodimeric glycyl-tRNA synthetase (GlyRS) are casually linked to Charcot-Marie-Tooth disease, the most common peripheral neuropathy (16). A detailed analysis in the light of a high-resolution structure showed that these mutations are in and around the dimer interface (36). In another example, homodimeric human lysyl-tRNA synthetase (LysRS), which is packaged into the HIV virion with the tRNA\textsuperscript{ys3} primer (for reverse transcription) and the Gag pro-
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The use of a monomer-dimer equilibrium to regulate cytokine function is universal and therefore was not the selective pressure for dimer formation. Instead, a pre-existing monomer-dimer equilibrium was adopted for a regulatory role, at the time of insects, when the critical (for cytokine signaling) ELR motif and EMAP II-like C-domain were simultaneously incorporated into TyrRS (11). As stated above, the dimer interface of human TyrRS is looser than that of a bacterial ortholog. Our calculated buried surface area from formation of the homodimer is 1129 and 1392 Å² for human (22) and Bacillus stearothermophilus TyrRS (40), respectively.⁴ Possibly, the development of a looser dimer interface that enabled a more facile monomer-dimer equilibrium may have occurred at the same time as the adoption of the ELR motif and EMAP II-like C-domain.

By analogy with \( k_{cat} \) and \( K_m \) parameters for catalysis of aminoacylation, the antagonist activity of the TyrRS\(^{Mini} \) dimer suggests that the synthetase has \( k_{cat} \) (for signaling) and \( K_m \) (for binding) parameters for its interaction with the CXCR1/2 receptors. The concentration dependence of the inhibition of the monomer-induced cell migration by the dimer suggests that the apparent \( K_m \) values for the monomer and dimer are similar (about 10–50 nM (Fig. 3b)). In contrast, to achieve the apparent complete inactivity of the dimer in the cell migration assay (Fig. 3a), we estimate that the \( k_{cat} \) for signaling by the dimer must be reduced at least 10-fold as compared with that of the monomer to make it an operational antagonist. Possibly, the dimer interface has determinants that block a conformational change needed for activation of signaling after TyrRS\(^{Mini} \) is bound to CXCR1/2.

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