

Cys-tRNA^{Pro} editing by *Haemophilus influenzae* YbaK via a novel synthetase/YbaK/tRNA ternary complex*

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Running Title: *Trans*-editing by a ProRS/YbaK/tRNA^{Pro} complex

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Aminoacyl-tRNA synthetases are multi-domain enzymes that often possess two activities to ensure translational accuracy. A synthetic active site catalyzes tRNA aminoacylation, while an editing active site hydrolyzes mischarged tRNAs. Prolyl-tRNA synthetases (ProRS) have been shown to misacylate Cys onto tRNA^{Pro}, but lack a Cys-specific editing function. The synthetase-like *Haemophilus influenzae* YbaK protein was recently shown to hydrolyze misacylated Cys-tRNA^{Pro} *in trans*. However, the mechanism of specific substrate selection by this single-domain hydrolase is unknown. Here, we demonstrate that YbaK alone appears to lack specific tRNA recognition capabilities. Moreover, YbaK cannot compete for aminoacyl-tRNAs in the presence of elongation factor Tu, suggesting that YbaK acts before release of the aminoacyl-tRNA from the synthetase. In support of this idea, cross-linking studies reveal the formation of binary (ProRS/YbaK) and ternary (ProRS/YbaK/tRNA) complexes. The binding constants for the interaction between ProRS and YbaK are 550 nM and 45 nM in the absence and presence of tRNA^{Pro}, respectively. These results suggest that the specificity of *trans*-editing by YbaK is ensured through formation of a novel ProRS/YbaK/tRNA complex.

Aminoacyl-tRNA synthetases maintain the high fidelity of protein synthesis by activating specific amino acids and transferring them to cognate tRNAs in a process known as aminoacylation. However, some synthetases are known to misactivate noncognate amino acids, transferring them onto their cognate tRNAs. Therefore, to ensure accurate translation of the genetic code, these enzymes, which include

members of both classes of synthetases, have evolved proofreading or editing functions (1-17). Pretransfer editing involves hydrolysis of the misactivated aminoacyl-adenylate, whereas posttransfer editing refers to specific hydrolysis of misacylated tRNA.

To clear noncognate amino acids, a double-sieve mechanism was proposed, wherein larger amino acids are rejected by the aminoacylation active site (i.e., coarse sieve) while smaller amino acids are cleared in a second editing active site (i.e., fine sieve) (18,19). This hypothesis was confirmed by more recent biochemical and X-ray crystallographic studies (20). Although the exact site of pretransfer editing is still an open question in most systems (21), class I editing enzymes, including isoleucyl-tRNA synthetase, valyl-tRNA synthetase, and leucyl-tRNA synthetase, use the highly conserved connective polypeptide 1 domain to edit misacylated tRNAs (1,2,4-8,12). In contrast, the posttransfer editing domains used by class II synthetases are much more diverse. The internal editing domain of alanyl-tRNA synthetase (AlaRS)¹ and the N-terminal editing domain of threonyl-tRNA synthetase (with the exception of the archaeobacterial enzymes) share sequence similarity (9,22), while the editing domains of bacterial prolyl-tRNA synthetase (ProRS) (13) and phenylalanyl-tRNA synthetase (17) appear to be unique, sharing no sequence homology to any of the other known synthetase editing domains. A distinct domain for editing has recently been identified in archaeobacterial threonyl-tRNA synthetases as well (22,23).

Most bacterial ProRSs contain a unique domain inserted between the class II consensus motifs, which is responsible for Ala-specific posttransfer editing activity (13,14,16). However, although ProRSs from all three kingdoms of life activate and aminoacylate noncognate Cys *in vitro* (14,24-26), they lack a Cys-specific editing

activity (24,26,27). Thus, in the ProRS system, a double-sieve mechanism does not appear to be sufficient to remove Cys-tRNA^{Pro}.

Recently, we showed that the *Haemophilus influenzae* YbaK protein, which shares significant sequence and structural homology to the Ala-specific editing domain of bacterial ProRSs, possesses posttransfer editing activity *in vitro* (16,28). Interestingly, this free-standing editing domain has an altered substrate specificity relative to ProRS. Whereas the full-length synthetase edits Ala-tRNA^{Pro}, the YbaK protein hydrolyzes Cys-tRNA^{Pro}. These data support at least one mechanism by which cells can survive despite the capability of ProRSs to misacylate Cys onto tRNA^{Pro} (14,24-26). These results led to the proposal that a triple-sieve mechanism of editing may be used to clear Cys-tRNA^{Pro} in at least some organisms, with the YbaK protein functioning as a third sieve *in trans* (28). A recent study showed that mischarged Cys-tRNA^{Pro} species indeed form in *Escherichia coli* cells, and that the YbaK protein possesses Cys hydrolysis activity *in vivo* (29). However, many open questions remain regarding the mechanism of this novel *trans*-editing function. For example, whether YbaK acts alone or in complex with aminoacyl-tRNA synthetases is unknown.

Here, we continue to probe the substrate and co-factor requirements for specific deacylation by *H. influenzae* YbaK. We demonstrate that, in the absence of other factors, all Cys-tRNA species tested are edited by YbaK, including Cys-tRNA^{Cys}. However, *in vitro* kinetic data suggest that the rate of Cys-tRNA^{Cys} hydrolysis by YbaK is significantly lower than the rate of Cys-tRNA^{Cys} synthesis by cysteinyl-tRNA synthetase (CysRS). In contrast, YbaK hydrolyzes Cys-tRNA^{Pro} at a rate that exceeds Cys-tRNA^{Pro} synthesis by ProRS. Moreover, fluorescence anisotropy measurements and chemical cross-linking studies support the formation of a novel ProRS/YbaK/tRNA^{Pro} *trans*-editing complex. Therefore, editing of Cys-tRNA^{Pro} is likely to be achieved *in vivo* via specific interactions between YbaK and ProRS/tRNA^{Pro}. This is the first demonstration that a free-standing editing protein forms a complex with a synthetase/tRNA pair, and provides a model for understanding how specific substrate selection is achieved by a single-domain editing module.

EXPERIMENTAL PROCEDURES

Materials – All amino acids and chemicals were purchased from Sigma unless otherwise noted. [³H]-Alanine (54 Ci/mmol) and [³H]-proline (99 Ci/mmol) were from Amersham Biosciences and [³⁵S]-cysteine (1075 Ci/mmol) was from PerkinElmer Life Sciences. All tRNAs used in this study were prepared by *in vitro* transcription, as described before (11,30-32).

Enzyme preparation – Wild-type (WT) *E. coli* ProRS and AlaRS, and WT *Methanococcus jannaschii* ProRS were purified using the Talon cobalt affinity technique (Clontech) as previously described (33). WT *H. influenzae* YbaK protein was purified using the IMPACTTM I system (New England Biolabs) as described (16,34). The *H. influenzae* K46A YbaK mutant was prepared using Ni²⁺-NTA resin (Qiagen) as described before (28). *E. coli* elongation factor-Tu (EF-Tu) was isolated in the EF-Tu·GDP form from *E. coli* strain DH10B-T1 (Invitrogen) carrying pPROEX-HTb_EF-Tu (35,36). The protein was purified using Ni²⁺-NTA resin (Qiagen), followed by removal of the His-tag using the AcTEV protease (Invitrogen). In the case of *E. coli* CysRS, plasmid pET-22b_CysRS was transformed into BL21(DE3) (Novagen), and the protein was purified using the Talon cobalt affinity resin (Clontech) as previously described (33). The concentrations of *E. coli* EF-Tu, *M. jannaschii* ProRS and *H. influenzae* YbaK proteins were determined by the Bradford assay (37). The concentrations of *E. coli* ProRS, CysRS and AlaRS were determined using the active site titration assay (38).

E. coli EF-Tu·GDP was converted into an active EF-Tu·GTP complex as previously reported (39-41). Briefly, purified EF-Tu·GDP (5 μM) was incubated at 37 °C in buffer containing 50 mM HEPES (pH 7.0), 150 mM NH₄Cl, 20 mM MgCl₂, 5 mM dithiothreitol (DTT), 10 μM GTP, 3 mM phosphoenolpyruvate and 30 μg/ml pyruvate kinase. The incubation was performed for 3 hours prior to use in aminoacylation and deacylation assays.

Aminoacylation assays and preparation of aminoacyl-tRNAs – Aminoacylation assays with [³H]-amino acids were performed at room temperature as previously described using purified

tRNA transcripts prepared *in vitro* (15,31,42). *E. coli* AlaRS (0.1 μM) was used to aminoacylate *E. coli* tRNA^{Ala} (4.0 μM) in the presence of both [³H]-Ala (5.3 μM) and cold Ala (20 μM) in buffer containing 50 mM HEPES (pH 7.5), 4 mM ATP, 20 mM KCl, 20 mM β -mercaptoethanol, 25 mM MgCl₂, and 0.1 mg/ml bovine serum albumin (BSA). For [³⁵S]-Cys aminoacylation reactions, *E. coli* CysRS (4 nM) was used to aminoacylate Cys onto *E. coli* tRNA^{Cys} (5 μM) with [³⁵S]-Cys (0.9 μM) and cold Cys (50 μM) in reaction buffer containing 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM MgCl₂, 25 mM DTT and 2 mM ATP as described previously (28,30).

[³⁵S]-Cys was mischarged onto *E. coli* G1 tRNA^{Pro} (8 μM), *E. coli* tRNA^{Cys} (8 μM) and *M. jannaschii* tRNA^{Pro} (8 μM) using *E. coli* ProRS (8 μM), *E. coli* CysRS (0.7 μM) and *M. jannaschii* ProRS (10 μM), respectively, in the [³⁵S]-Cys buffer described above. The mischarged tRNAs were purified by repeated phenol extractions, followed by ethanol precipitation. The aminoacyl-tRNAs were quantified by scintillation counting and stored at -20 °C in 50 mM KPO₄ (pH 5.0).

Deacylation assays – Deacylation assays were carried out at room temperature according to published conditions (11,25). Reactions contained approximately 1.0 μM *E. coli* [³⁵S]-Cys-tRNA^{Cys}, 0.1 μM *E. coli* [³⁵S]-Cys-tRNA^{Pro}, 0.1 μM *E. coli* [³⁵S]-Cys-G1 tRNA^{Pro} or 0.3 μM *M. jannaschii* [³⁵S]-Cys-tRNA^{Pro}. Reactions were initiated with the amount of protein indicated in the figure legends. In each case, a background reaction was carried out in which buffer (0.15 M KPO₄, pH 7.0) was used to initiate the reaction. Although for clarity only one representative data set is shown in the figures, a separate background reaction was performed for each aminoacyl-tRNA species. First order deacylation rate constants (k_{obs}) were calculated according to $k_{\text{obs}} = \ln 2/t_{1/2}$ (43). The experimentally determined half-lives, $t_{1/2}$, represent the average of at least three independent trials.

Fluorophore labeling of *H. influenzae* YbaK protein – A solution of Alexa Fluor® (AF) 488-tetrafluorophenyl ester (Molecular Probes, Eugene, OR) was prepared in distilled DMSO, and the concentration was determined using the extinction coefficient supplied by the manufacturer ($\epsilon_{494} =$

71,000 cm⁻¹M⁻¹). The YbaK protein stock was desalted by elution through a MicroSpin™ Sephadex G25 column (Amersham) pre-equilibrated in 40 mM HEPES (pH 7.5) and 50 mM NaCl. The protein (40 μM) was incubated with 10-fold molar excess of AF for 15 min at room temperature in the same buffer. Unreacted dye was immediately removed by elution through a MicroSpin™ Sephadex G25 column. The labeled protein, YbaK-AF, was applied to a Microcon YM-10 concentrator (Amicon) to remove residual free dye and to exchange into the following buffer: 10 mM HEPES (pH 8.0), 0.25 M NaCl, 0.05 mM EDTA, 0.05% Triton, 5 mM DTT and 40% glycerol. The labeled protein was visualized on a 15% SDS-polyacrylamide gel and its final concentration was determined by the Bradford assay (37). The labeling stoichiometry was determined by the following equation: $R = [A_{494} X (\text{dilution factor})] / [\epsilon_{494} X Y]$, where R is the molar ratio of dye to YbaK, A_{494} represents the absorbance of the labeled protein solution at 494 nm, ϵ_{494} is the extinction coefficient of the dye, and Y is the final concentration of YbaK-AF determined by the Bradford assay (37). Prior to use in fluorescence anisotropy measurements, the activity of YbaK-AF was verified by standard deacylation assays using Cys-tRNA^{Pro} as a substrate (28).

Fluorescence anisotropy measurements – Equilibrium dissociation constants were determined by measuring the fluorescence anisotropy of YbaK-AF as a function of increasing concentrations of an unlabeled protein or protein-tRNA complex. YbaK-AF (50 nM) was incubated with varying amounts of the desired unlabeled protein (or protein-tRNA complex) for 30 min at room temperature in buffer containing 40 mM HEPES (pH 7.5) and 50 mM NaCl. The following concentration ranges were used: *E. coli* ProRS (40 nM to 8 μM), CysRS (15 nM to 10 μM), AlaRS (25 nM to 6 μM), ProRS/tRNA^{Pro} (40 nM to 3 μM), CysRS/tRNA^{Cys} (10 nM to 2.2 μM), AlaRS/tRNA^{Ala} (25 nM to 6 μM) and BSA (40 nM to 8 μM). The synthetase-tRNA complexes were pre-incubated for 30 min at room temperature prior to incubation with YbaK-AF. Anisotropy measurements were made on a PTI spectrofluorimeter (Model QM-2000; Photon Technology International, Lawrenceville, NJ). The

excitation and emission wavelengths were 490 nm and 520 nm, respectively (slit widths = 5 nm). Anisotropy was measured using the time-based function for 30 sec (integration time = 1 sec; resolution = 8 sec) and the data were averaged. All measurements were carried out at least three times. The titration curves were fit to the following equation, which assumes a 1:1 binding stoichiometry (44-46):

$$A = A_{\min} + [(Y + S + K_d) - \{(Y + S + K_d)^2 - (4YS)\}^{1/2}](A_{\max} - A_{\min})/(2Y)$$

where A is the measured anisotropy at a particular total concentration of the unlabeled protein or protein-tRNA complex (S) and the AF-labeled YbaK protein (Y), A_{\min} is the minimum anisotropy, A_{\max} is the final maximum anisotropy, and K_d is the dissociation constant.

Cross-linking analysis – Cross-linking reactions were carried out in a total volume of 20 μ L in buffer containing 150 mM NaCl and 15 mM sodium phosphate (pH 7.5). *E. coli* ProRS (2.5 μ M), *H. influenzae* YbaK (20 μ M) and *E. coli* tRNA^{Pro} (20 μ M) were pre-incubated for 15 min at room temperature, followed by addition of Ru(bpy)₃Cl₂ (125 μ M) (Fluka) and ammonium persulfate (2.5 mM) in the dark. Samples were irradiated with a flashlight for 1 min at 7 cm distance. Reactions were quenched with protein loading buffer containing 50 mM Tris (pH 6.7), 100 mM β -mercaptoethanol, 2% SDS, 10% glycerol and 0.1% bromophenol blue. After heating for 5 min at 80 $^{\circ}$ C, samples were loaded onto 10% SDS-polyacrylamide gels and visualized using Coomassie Blue stain. Some experiments were carried out using [³²P]-labeled tRNA^{Pro} prepared by *in vitro* transcription using [³²P]- α -GTP and visualized using phosphorimaging. In addition to reactions containing all three molecules (i.e., ProRS, YbaK, and tRNA), two-component (i.e., ProRS and YbaK, ProRS and tRNA, and YbaK and tRNA) and single component cross-linking reactions were also carried out. In some experiments, *E. coli* CysRS or BSA were substituted for ProRS. Negative control experiments were performed in the absence of one of the three components required for the cross-linking reaction (i.e., visible light, Ru(bpy)₃Cl₂, or ammonium persulfate).

Two-dimensional electrophoresis – The ProRS-YbaK-tRNA^{Pro} cross-linking reaction was performed as described above, except that the quenching step was performed with only 100 mM β -mercaptoethanol. The sample was then subjected to 2D electrophoresis. Briefly, the sample was applied onto an Immobiline DryStrip gel (13 cm or 24 cm, Amersham) to separate proteins based on their isoelectric points (between pH 3 to 10) using the Ettan IPGphor Isoelectric Focusing System (Amersham). The strip was then loaded onto a precast 4 to 20% gradient SDS-polyacrylamide gel (Bio-Rad) for separation in the second dimension. Bands were visualized using the Deep PurpleTM Total Protein Stain (Amersham). This work was performed at the Mass Spectrometry Consortium for the Life Sciences and Protein Analysis Facility at University of Minnesota.

In-gel tryptic digestion and mass spectrometry analysis – Following cross-linking, the desired bands from the polyacrylamide gel (1D or 2D) were cut out and soaked in buffer (80 μ L) containing 50 mM NH₄HCO₃ (pH 8.5), 5 mM CaCl₂ and 12.5 ng/ μ L trypsin (Promega). After incubation on ice for one hour, excess liquid was removed and 80 μ L of buffer containing 50 mM NH₄HCO₃ (pH 8.5) and 5 mM CaCl₂ was added. The reaction tube was incubated for 16 hours at 37 $^{\circ}$ C. After removing excess liquid, incubation in 100 μ L of 20 mM NH₄HCO₃ (pH 8.5) was carried out for 20 min at 37 $^{\circ}$ C. Peptides were extracted from the gel piece using a freshly prepared 5% formic acid solution in 50% CH₃CN. For mass spectrometry analysis, the sample was desalted using a PepClean C18 spin column (Pierce). The desalted sample was lyophilized and submitted for electrospray ionization mass spectrometry analysis carried out at the Mass Spectrometry Consortium for the Life Sciences and Protein Analysis Facility (University of Minnesota). Obtained product ion mass spectra were searched using the following software package/database: ProID (ABI)/NCBI's non-redundant database. Mass accuracy was calculated as the following: Accuracy (ppm) = { |(Theoretical m/z value) – (Experimental m/z value)| / (Theoretical m/z value)} X 10⁶.

RESULTS

H. influenzae YbaK does not recognize the first base pair of the acceptor stem – We have previously reported that *H. influenzae* YbaK rapidly deacylates *E. coli* Cys-tRNA^{Pro}, but only weakly hydrolyzes an Ala-tRNA^{Pro} G1:C72/U70 variant as well as Ser- and Gly-tRNA^{Ala} (28). Bacterial tRNA^{Pro} species contain a highly conserved C1:G72 base pair (47,48), and this unique sequence element plays a critical role in tRNA^{Pro} recognition by *E. coli* ProRS (31,48). This prompted us to investigate whether the C1:G72 base pair is part of the signal that marks tRNA^{Pro} as a preferred substrate for YbaK.

First, we misacylated Cys onto a tRNA^{Pro} variant containing a C1→G1 substitution, which was accomplished using *E. coli* ProRS. Additionally, Cys was mischarged onto *M. jannaschii* tRNA^{Pro} using *M. jannaschii* ProRS, because this tRNA contains a G1:C72 base pair. Deacylation assays with both of these Cys-tRNA species show that they are readily deacylated by YbaK (Figure 1). Furthermore, we also charged Cys onto *E. coli* tRNA^{Cys} using *E. coli* CysRS. This tRNA differs from *E. coli* tRNA^{Pro} at five positions at the top sequence of the acceptor stem, including the first base pair (G1:C72) and the discrimination base (U73). Despite these differences, Cys-tRNA^{Cys} was a good substrate for YbaK (Figure 1), although the rate of deacylation ($k_{\text{obs}} = 0.001 \text{ s}^{-1}$) was ~3-fold reduced relative to deacylation of the tRNA^{Pro} substrates ($k_{\text{obs}} = 0.0028 \text{ s}^{-1}$). Thus, YbaK does not use the C1:G72 base pair of tRNA^{Pro} as a positive recognition element, nor does it use the G1:C72 pair or U73 of tRNA^{Cys} for negative discrimination. Based on this work and a recent report wherein all twenty cognate aminoacyl-tRNA species were examined as substrates for YbaK (29), this deacylase appears to preferentially recognize Cys-tRNA species independent of the first base pair or the discriminator base, while also displaying weak hydrolysis activity in the presence of a subset of other aminoacyl-tRNAs.

Specific Cys-tRNA^{Pro} deacylation occurs in the presence of prolyl-tRNA synthetase – If the YbaK protein is truly involved in maintaining the high fidelity of protein synthesis *in vivo* by acting as a *trans*-editing protein, how can we explain the observed hydrolysis of Cys-tRNA^{Cys} *in vitro*? We hypothesize that the presence of other factors

absent in our *in vitro* assays may help to ensure that YbaK carries out specific hydrolysis of Cys-tRNA^{Pro} *in vivo*. To probe this hypothesis, we tested the substrate specificity of *H. influenzae* YbaK in the presence of *E. coli* EF-Tu and various aminoacyl-tRNA synthetases.

To establish the influence of EF-Tu on the specificity of aminoacyl-tRNA selection by YbaK, purified *E. coli* EF-Tu·GDP was first converted into an active EF-Tu·GTP complex (39-41). In the presence of one equivalent EF-Tu·GTP (0.3 μM), the deacylation activity of the YbaK protein (0.3 μM) was completely abolished using both Cys-tRNA^{Pro} (Figure 2A) and Cys-tRNA^{Cys} (Figure 2B) as substrates. As a control, in the presence of the inactive EF-Tu·GDP (0.3 μM) complex, YbaK displayed full hydrolysis activity (data not shown). Therefore, an active EF-Tu·GTP complex appears to protect the Cys-tRNA linkage and prevent hydrolysis by YbaK. Although the binding of Cys-tRNA^{Pro} to EF-Tu·GTP has to our knowledge not been examined, the active EF-Tu·GTP complex is known to bind a number of mischarged tRNA species with nanomolar affinity (49).

We had previously reported that a 4-fold excess of YbaK over ProRS was sufficient to significantly decrease the rate of Cys-tRNA^{Pro} synthesis *in vitro* (28). Based on this study, it appeared that YbaK hydrolyzed Cys-tRNA^{Pro} synthesized by ProRS *in situ* or, alternatively, that the presence of YbaK prevented tRNA^{Pro} misacylation with Cys. To distinguish between these two possibilities, we carried out a similar study with a K46A YbaK point mutant. Mutation of the strictly conserved Lys46 residue was previously shown to abolish posttransfer editing by YbaK (28). A 4-fold excess of K46A YbaK did not affect Cys-tRNA^{Pro} synthesis (Figure 3A). In contrast, a similar amount of wild-type YbaK decreased the initial rate of aminoacylation by 4.3 fold, as expected based on previous results (Figure 3A) (28). These results support a model wherein tRNA^{Pro} is mischarged with Cys by ProRS followed by deacylation by YbaK.

To determine whether YbaK can efficiently compete for aminoacyl-tRNA binding in the presence of EF-Tu, we carried out a similar experiment to the one shown in Figure 3A, except this time a 4-fold excess of both EF-Tu·GTP and YbaK was added to the aminoacylation reaction. Under these conditions, YbaK did not catalyze

hydrolysis of Cys-tRNA^{Pro} (data not shown). Similarly, when ProRS, YbaK and EF-Tu•GTP were all pre-incubated with tRNA^{Pro} prior to initiating the aminoacylation reaction with ATP, YbaK did not catalyze hydrolysis of Cys-tRNA^{Pro} (data not shown). These results are consistent with previous studies of post-transfer editing of Val-tRNA^{Ile} by isoleucyl-tRNA synthetase (50). Taken together, these data suggest that post-transfer editing by aminoacyl-tRNA synthetases or synthetase-like proteins occurs prior to interacting with EF-Tu.

We next determined the effect of YbaK on the synthesis of Cys-tRNA^{Cys} by *E. coli* CysRS. In sharp contrast to the results obtained with ProRS, the presence of YbaK did not reduce the level of Cys-tRNA^{Cys} formation even at a CysRS:YbaK ratio of 1:25 (Figure 3B). Interestingly, addition of YbaK appears to protect the aminoacyl linkage, resulting in higher levels of Cys charging than in the absence of YbaK. Because the CysRS assays require much lower concentrations of synthetase than the ProRS assays, lower YbaK concentrations were required in the former case to achieve the desired synthetase:YbaK ratio. Therefore, we also tested the effect of addition of high concentrations of YbaK (5 μ M) on Cys-tRNA^{Cys} formation by *E. coli* CysRS (2 nM). The initial rate of Cys-tRNA^{Cys} formation by *E. coli* CysRS under these low enzyme conditions was comparable to the rate of Cys-tRNA^{Pro} formation by *E. coli* ProRS (28). As shown in Figure 3B (inset), addition of 2500-fold excess of YbaK over CysRS resulted in an even greater stimulation of Cys-tRNA^{Cys} formation than the lower concentrations of YbaK. Using conditions more similar to those previously used to measure initial rates of Cys-tRNA^{Cys} synthesis by CysRS (30 nM) (30), the presence of 25-fold excess YbaK (0.75 μ M) also stimulated the initial rate of Cys-tRNA^{Cys} synthesis (data not shown). Although the mechanism of stimulation of Cys-tRNA^{Cys} synthesis by YbaK is currently unknown, these data demonstrate that the rate of Cys-tRNA^{Cys} synthesis in the presence of YbaK is much greater than the rate of deacylation by YbaK (Figure 1), suggesting that the Cys-tRNA^{Cys} deacylation activity may not be relevant *in vivo*.

To determine whether the negative and positive effects of YbaK on cysteinylolation by ProRS and CysRS, respectively, are specific to these two systems, we performed similar

experiments to test YbaK's effect on cognate aminoacylation by *E. coli* ProRS and AlaRS. In contrast to the results shown in Figure 3, excess YbaK (1 μ M) had no effect on cognate prolylation or alanylation by ProRS or AlaRS (0.1 μ M), respectively. We also showed that addition of a non-specific protein such as BSA has no effect on cysteinylolation by CysRS (data not shown). Thus, the effects reported in Figure 3 appear to be specific.

Binding affinity of YbaK to ProRS is increased in the presence of tRNA^{Pro} – The biochemical assays described above suggest that the substrate specificity of *trans*-editing by YbaK may be modulated through specific interactions with aminoacyl-tRNA synthetases. To test this hypothesis, the ProRS/YbaK interaction was characterized using fluorescence anisotropy to measure the equilibrium binding constant (K_d) both in the absence and presence of tRNA^{Pro}. In these experiments, YbaK was labeled with an amino-reactive extrinsic fluorophore, Alexa Fluor (AF) 488-tetrafluorophenyl ester. The reaction conditions were chosen such that the ratio of dye to YbaK was kept low (0.2 to 0.6). The labeled protein, YbaK-AF, showed a similar rate of Cys-tRNA^{Pro} deacylation as unlabeled YbaK (data not shown).

We first investigated the binding affinity between YbaK-AF and ProRS in the absence of tRNA (Figure 4A). Titration of YbaK-AF with *E. coli* ProRS resulted in a significant increase in anisotropy and a binding constant of 550 nM was measured (Figure 4A and Table I). The affinity between YbaK and ProRS increased 12-fold in the presence of cognate tRNA^{Pro} (45 nM), providing strong support for a direct interaction between these three species (Figure 4B and Table I). Although it would be of great interest to measure the binding affinity in the presence of both properly charged and mischarged tRNA, high-levels of deacylation under the conditions of the binding assay made these experiments technically unfeasible. Binding between YbaK and CysRS was also detected using this approach, with K_d values of 404 nM and 165 nM measured in the absence and presence of tRNA^{Cys}, respectively (Table I). These results are in accordance with the biochemical studies, showing that the presence of YbaK stimulates CysRS aminoacylation activity

(Figure 3B). In contrast, binding to AlaRS was extremely weak and only a lower limit for the K_d could be estimated based on the fluorescence anisotropy data (> 2000 nM) (Table I). Moreover, addition of tRNA^{Ala} did not appear to increase the affinity in this case.

Cross-linking analysis identifies a

ProRS/YbaK/tRNA ternary complex –We also carried out a cross-linking study using the zero-length oxidative cross-linking reagent, tris(2, 2'-bipyridyl)ruthenium(II) chloride (Ru(bpy)₃Cl₂), in the presence of ammonium persulfate and visible light (51). This method has several advantages over other chemical cross-linking approaches for probing the architecture of protein-protein complexes (51,52). This technique uses a photo-generated oxidant to mediate rapid and efficient cross-linking using visible light (i.e., an ordinary flashlight), resulting in high yields of cross-linked products with irradiation times of <1 min. The activated metal complex, Ru(III)(bpy)₃³⁺, is believed to extract an electron from Tyr or Trp, leading to radical species in proteins, which can couple only intimately associated proteins *via* this zero-length chemistry (51). Although this method was used by numerous labs for the analysis of protein-protein interactions ((52) and refs therein), protein-DNA cross-linking *via* guanine oxidation has also been shown to occur (53).

In our application, various combinations of ProRS, YbaK and tRNA^{Pro} were subjected to similar cross-linking conditions. The result of one such assay is shown in Figure 5. In the presence of ProRS alone (Figure 5A, lane 2), higher-order complexes (labeled a) are formed, most likely due to monomer-monomer cross-linking between subunits in homodimeric ProRS. In the presence of ProRS and YbaK (Figure 5A, lane 3), a new band (labeled c) appears, which may represent formation of a ProRS/YbaK complex. Cross-linking of ProRS and tRNA (Figure 5A, lane 4) also produces a band that co-migrates with band c, as well as an additional weak band labeled b. Upon incubation of all three molecules, complexes that co-migrate with bands b and c are observed (Figure 5A, lane 5). In this case, we also observe a reduction in the formation of higher molecular mass species (bands a) and an enhancement of the ProRS monomer band (labeled d).

To identify the components of the cross-linked complexes, band b (Figure 5A, lane 5) and band c (Figure 5A, lanes 3, 4 and 5) were subjected to in-gel trypsin digestion followed by liquid chromatography (LC)-electrospray ionization (ESI) mass spectrometry analysis. This analysis showed that band c in lanes 3 and 5 contained both ProRS and YbaK proteins. Briefly, ProID sequence analysis revealed 22.6% (lane 3) and 49.7% (lane 5) sequence coverage for *E. coli* ProRS, and 17.7% (lane 3) and 36.1% (lane 5) sequence coverage for *H. influenzae* YbaK. Based on the mass accuracy (ppm) and MS/MS analysis of the identified peptides, it is clear that band c in both lanes 3 and 5 contains a ProRS/YbaK conjugate. As expected, band c in lane 4 contains only *E. coli* ProRS in addition to tRNA, which was identified by carrying out an independent cross-linking experiment using [³²P]-labeled tRNA (data not shown). [³²P]-Labeled tRNA was also observed in band b of lane 4, likely reflecting a complex consisting of more than one tRNA molecule. Two-dimensional (2D) electrophoresis (Figure 5B) followed by mass spectrometry analysis also confirmed that band c in lane 5 contains several species, including a ProRS/YbaK complex.

In addition to bands b and c in lane 4, experiments carried out with [³²P]-tRNA indicated the presence of tRNA in bands b and c of lane 5 (Figure 5A). Mass spectrometry analysis of band b in lane 5 also revealed peptides that originated from both *E. coli* ProRS (52.4% sequence coverage) and *H. influenzae* YbaK (43.0% sequence coverage). To verify that band b contains a ternary complex, 2D electrophoresis was performed, revealing that band b contains two major species (Figure 5B and 5C). Mass spectrometry analysis showed that each band contains both ProRS and YbaK. Since band b in the 1D analysis (Figure 5A) contained [³²P]-tRNA, at least one of the species shown in Figure 5C must be a ternary complex. The separation into two distinct bands may be due to the presence of complexes with different stoichiometries of the three interacting molecules. Alternatively, one of the species may represent a binary complex. When a similar cross-linking analysis was carried out with BSA or *E. coli* CysRS rather than ProRS, no distinct complexes that migrated slower than the BSA or CysRS bands were detected by Coomassie Blue staining. We also confirmed that the species

we identified as cross-linked complexes in the experiments with ProRS depended on the presence of each of the components required for the oxidative cross-linking reaction (i.e., visible light, Ru(bpy)₃Cl₂, and ammonium persulfate).

DISCUSSION

Aminoacyl-tRNA synthetases are modular enzymes (54,55) containing an ancient catalytic core responsible for adenylate synthesis and tRNA aminoacylation, and an anticodon binding domain fused to the N- or C-terminal side of the catalytic core. Additional domains that enhance the specificity of the synthetase are often found as insertions in the synthetic active site, or as extensions on the N- or C-terminus (56). Based on the ancient nature of synthetases and their key function in the development of the genetic code, it is not surprising that synthetase-like proteins are found widely distributed throughout the genomes of Bacteria, Eukarya, and Archaea (57,58). These proteins generally consist of a catalytic core domain only, and lack the additional modules characteristic of full-length synthetases.

While the role of many of these independent synthetase-like domains has been shown to be unrelated to protein translation (57,58), several, including the YbaK protein studied in this work, have been reported to perform aminoacylation or editing reactions *in trans* (15,16,23,28,29,59,60). However, since these free-standing domains do not contain extra modules to assist with tRNA binding, it is unclear how specific substrate selection is achieved. Indeed, we show here that unlike prokaryotic ProRS, the *H. influenzae* YbaK protein does not rely on either the first base pair or the discriminator base of the tRNA^{Pro} acceptor stem to recognize its aminoacyl-tRNA substrate. All Cys-tRNA species tested to date, including Cys-tRNA^{Cys}, have been shown to be substrates for deacylation by YbaK *in vitro* (Figure 1) (28,29).

In addition to the lack of specific tRNA recognition capabilities, channeling of aminoacyl-tRNAs is a well-documented phenomenon (61,62), and it is unlikely that free-standing charged tRNAs are ever present in the cell and available for interaction with *trans*-editing domains such as YbaK. Thus, one mechanism that synthetase-like proteins may use to carry out their specific

functions in the cell, involves interaction with protein/tRNA complexes.

E. coli EF-Tu is known to protect the aminoacyl-tRNA linkage prior to tRNA docking on the ribosome (49,63). An active EF-Tu·GTP complex also forms a tight complex with either correctly charged or mischarged aminoacyl-tRNA *in vitro* (49). Experiments with EF-Tu·GTP reported here (Figure 2 and data not shown) show that YbaK cannot compete for aminoacyl-tRNA binding in the presence of the elongation factor. Therefore, YbaK likely acts before release of the aminoacyl-tRNA from the synthetase, and EF-Tu interaction must occur at a step in the translation process that is distinct from the YbaK proofreading step.

To establish whether YbaK's specificity is modulated by the presence of aminoacyl-tRNA synthetases, we carried out aminoacylation assays with both *E. coli* ProRS and CysRS, respectively. Strikingly, the presence of wild-type YbaK results in hydrolysis of Cys-tRNA^{Pro} produced by ProRS, but does not negatively impact synthesis of Cys-tRNA^{Cys} by CysRS (Figure 3). In fact, in the presence of excess YbaK, increased levels of Cys-tRNA^{Cys} are observed. Although the mechanism of this stimulation is unknown and is the subject of ongoing investigations, this observation together with the significantly lower rate of YbaK deacylation of Cys-tRNA^{Cys} ($k_{\text{obs}} = 0.001 \text{ s}^{-1}$) compared to the rate of Cys-tRNA^{Cys} synthesis by *E. coli* CysRS ($k_{\text{cat}} = \sim 1.3 \text{ s}^{-1}$) (64), may explain why cognate Cys-tRNA^{Cys} hydrolysis by YbaK is not a significant problem for the cell.

In contrast, the rate of hydrolysis of Cys-tRNA^{Pro} by YbaK ($k_{\text{obs}} = 0.0028 \text{ s}^{-1}$) is greater than the rate of Cys-tRNA^{Pro} synthesis by *E. coli* ProRS ($k_{\text{cat}} = \sim 0.0004 \text{ s}^{-1}$; estimated from relative rates reported in (14,65)). Moreover, zero-length cross-linking analysis and binding measurements suggest that the specificity of Cys-tRNA^{Pro} discrimination by YbaK is ensured, in part, through the formation of a high-affinity ProRS/YbaK/tRNA^{Pro} complex (Figures 4 and 5; Table I). Although our cross-linking analysis could not detect CysRS/YbaK complex formation, fluorescence anisotropy measurements showed that YbaK also interacts with CysRS/tRNA^{Cys}, albeit with an ~ 4 -fold lower affinity than with ProRS/tRNA^{Pro} (Table I). The lack of cross-link formation using Ru(bpy)₃Cl₂ can be explained by

the absence of appropriately positioned Tyr or Trp residues.

Overexpression of YbaK has been shown to decrease (by 35%) *in vivo* levels of Cys-tRNA^{Cys} (29). However, the results reported here suggest that at least two factors contribute to specific deacylation of Cys-tRNA^{Pro} by YbaK, and provide an explanation for why this undesired Cys-tRNA^{Cys} hydrolysis activity does not affect cell growth under normal circumstances: (1) the relative rates of deacylation/synthesis, and (2) high-affinity ProRS-tRNA^{Pro} binding. The relative

rates of deacylation/synthesis favor Cys-tRNA^{Cys} synthesis and Cys-tRNA^{Pro} hydrolysis. Moreover, since YbaK does not appear to recognize specific sequence elements of its tRNA substrate and cannot compete effectively for aminoacyl-tRNA substrate binding in the presence of EF-Tu, formation of a ProRS/YbaK/tRNA^{Pro} ternary complex, is an alternative means to ensure specific recognition and hydrolysis of Cys-tRNA^{Pro} prior to its incorporation into the ribosomal protein synthesis machinery.

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FOOTNOTES

* We thank Dr. Ya-Ming Hou (Thomas Jefferson University) for kindly providing us with the expression plasmids for *E. coli* CysRS and tRNA^{Cys}, and Dr. Rachel Green (Johns Hopkins University) for supplying the *E. coli* EF-Tu expression plasmid. We would also like to acknowledge and thank Ms. Carmen Silvers for providing purified *M. jannaschii* ProRS and Ms. Byung Ran So for contributing to the deacylation studies of *M. jannaschii* Cys-tRNA^{Pro} by YbaK. We give special thanks to Dr. LeeAnn Higgins (University of Minnesota) for carrying out the mass spectrometry analysis, and Dr. Bruce Witthuhn (University of Minnesota) for performing the 2D-electrophoresis studies. This work was supported by grant GM49928 from the National Institutes of Health.

¹The abbreviations used are: AlaRS, alanyl-tRNA Synthetase; BSA, bovine serum albumin; CysRS, cysteinyl-tRNA Synthetase; EF-Tu, elongation factor-Tu; ProRS, prolyl-tRNA Synthetase.

FIGURE LEGENDS

FIG. 1. **Deacylation of aminoacyl-tRNAs by *H. influenzae* YbaK (1.0 μM).** The substrates were *E. coli* [³⁵S]-Cys-G1 tRNA^{Pro} (●), *E. coli* [³⁵S]-Cys-tRNA^{Cys} (▲), and *M. jannaschii* [³⁵S]-Cys-tRNA^{Pro} (■). The plots represent the average of at least three reactions with the standard deviations indicated. A background (no protein) reaction was performed for all tRNAs tested, and all were within 8% of the representative background data shown (□).

FIG. 2. **The influence of *E. coli* EF-Tu in deacylation of Cys-tRNAs by *H. influenzae* YbaK.** Deacylation of [³⁵S]-Cys-tRNA^{Pro} (A) or [³⁵S]-Cys-tRNA^{Cys} (B) by the YbaK protein (0.3 μM) in the absence (□) and presence (■) of *E. coli* EF-Tu-GTP (0.3 μM). The plots represent the average of at least three reactions with the standard deviations indicated. A background (no protein) reaction was performed for all tRNAs tested, and all were within 8% of the representative background data shown (○).

FIG. 3. **The effects of YbaK on cysteinylolation by *E. coli* ProRS and CysRS.** (A) Aminoacylation of tRNA^{Pro} (10 μM) with [³⁵S]-Cys by *E. coli* ProRS (0.5 μM) in the absence of YbaK (□) and in the presence of a 4-fold excess of *H. influenzae* wild-type YbaK (▲) or K46A YbaK protein (■). (B) Aminoacylation of tRNA^{Cys} (5 μM) with [³⁵S]-Cys by *E. coli* CysRS (4 nM) in the absence and presence of WT *H. influenzae* YbaK protein. Reactions were carried out in the absence of YbaK (□) and in the presence of 1:3 (■), 1:10 (●), 1:25 (◆) and 1:2500 (△, inset) ratios of CysRS to YbaK. The reactions shown in the inset were performed with 2 nM CysRS. The YbaK alone reaction contained 0.1 μM protein (▲). The plots represent the average of at least three reactions with the standard deviations indicated.

FIG. 4. **Fluorescence anisotropy experiments to determine the binding affinity of Alexa Fluor-labeled YbaK (YbaK-AF) to ProRS.** Measurements were carried out as a function of increasing concentrations of ProRS in the absence (A) and presence (B) of tRNA^{Pro}. The data were fit to the equation given in the Materials and Methods. Only a representative data set is shown, but all measurements were carried out at least three times with the equilibrium binding constants and standard deviations indicated in panels A and B (see also Table I).

FIG. 5. **Oxidative cross-linking analysis using Ru(bpy)₃Cl₂.** (A) Coomassie Blue stained 10% SDS-polyacrylamide gel showing protein markers (198 KDa, 144 KDa, and 96 KDa; lane 1), cross-linking of *E. coli* ProRS alone (lane 2), ProRS and *H. influenzae* YbaK (lane 3), ProRS and *E. coli* tRNA^{Pro} (lane 4), and ProRS, YbaK and tRNA^{Pro} (lane 5). The letters correspond to the major products as discussed in the

text. Asterisks (*) indicate bands containing radioactivity in experiments performed with [^{32}P]-labeled tRNA. (B) SDS-polyacrylamide gel showing the results of a 2D electrophoresis analysis of the reaction described in lane 5 of panel A. Only bands b and c are shown for clarity. (C) Lighter contrast of band b from panel B, showing that two major species are present.

TABLE I

*Equilibrium binding affinity constants (K_d) obtained from fluorescence anisotropy measurements using AlexaFluor 488 (AF)-labeled *H. influenzae* YbaK*

	No tRNA (nM)	+ Cognate tRNA (nM)
<i>H. influenzae</i> YbaK-AF	N.D. ¹	N.D. ²
YbaK-AF + <i>E. coli</i> ProRS	550 ± 70	45 ± 20
YbaK-AF + <i>E. coli</i> CysRS	404 ± 99	165 ± 23
YbaK-AF + <i>E. coli</i> AlaRS	> 2000	> 2000

¹N.D. = Not Detectable. No change in anisotropy was detected upon titration of YbaK-AF with protein storage buffer. ²No change in anisotropy was detected upon titration of YbaK-AF with *E. coli* tRNA^{Pro}.

FIG. 1

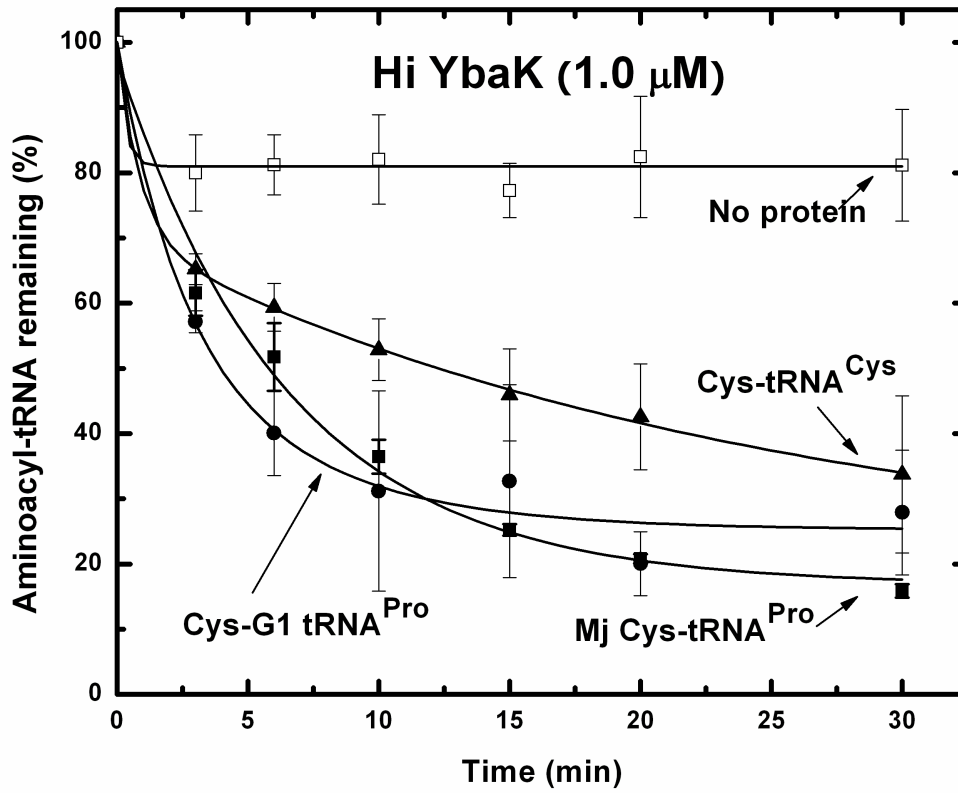


FIG. 2

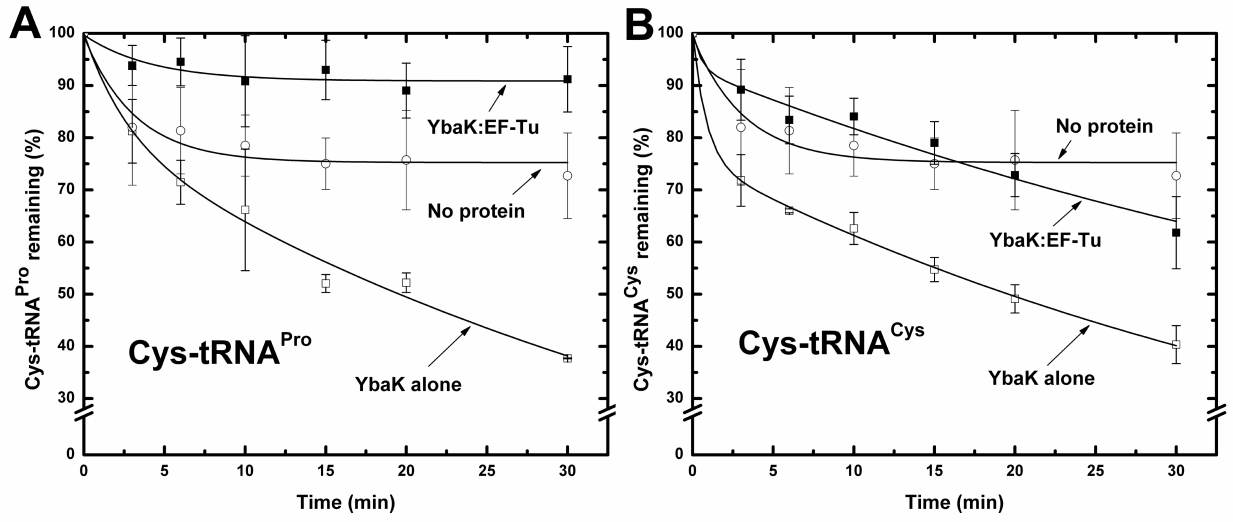


FIG. 3

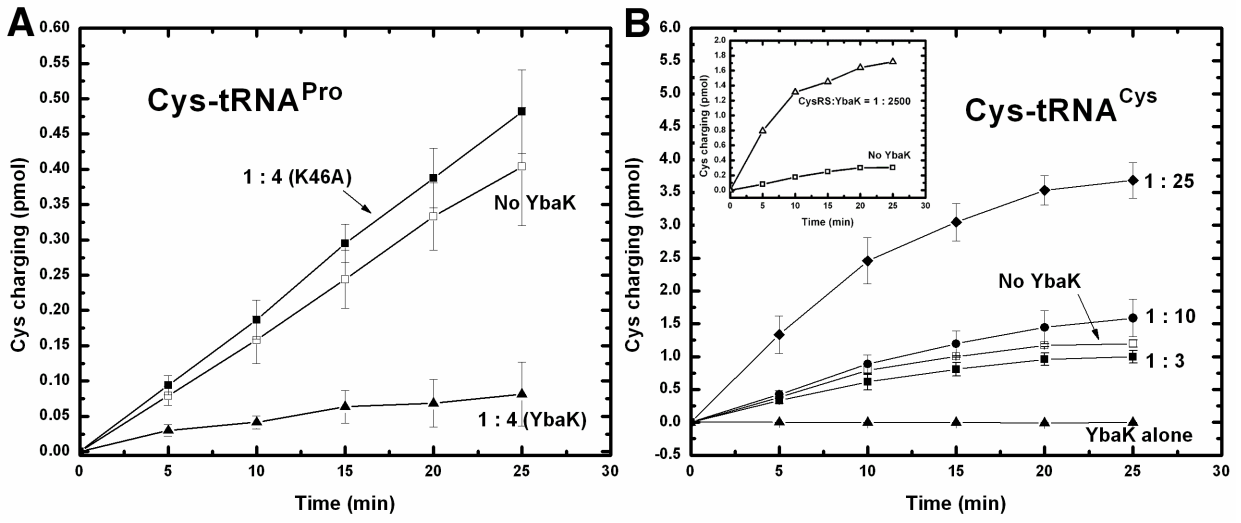


FIG. 4

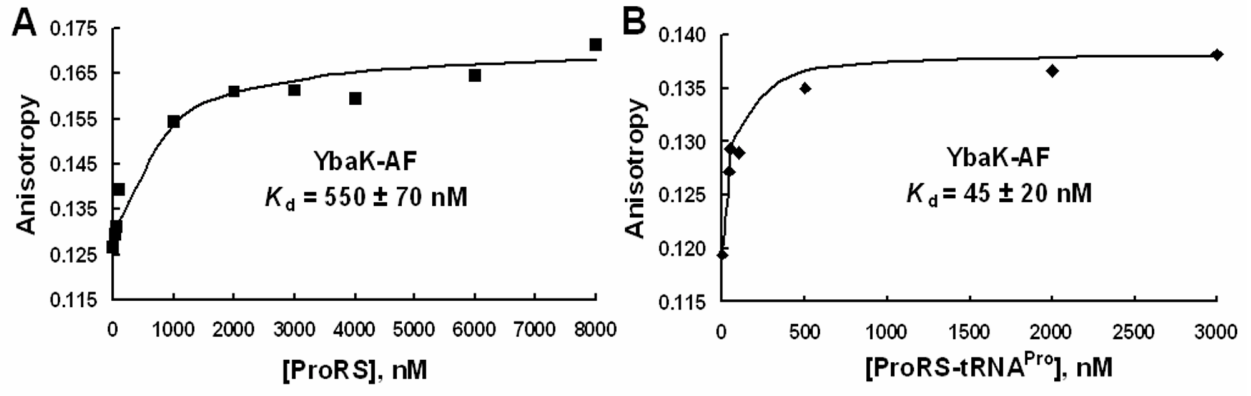


FIG. 5

