SUPPLEMENTARY INFORMATION

Aminoacyl-tRNA Substrate and Enzyme Backbone Atoms Contribute to Translational Quality Control by YbaK

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Supplementary Table 1: Calculated QM/MM energies of structures along the reaction coordinate in YbaK-catalyzed Cys-tRNA$^{Pro}$ hydrolysis. SV(P) energy represents structure optimization and energy calculation using the smaller SV(P) basis set. TZVPP energy represents structure optimization using the SV(P) basis set and energy calculation using the larger TZVPP basis set. Both methods yield similar results except the larger TZVPP basis set gives lower energy values for the product, likely due to better treatment of the proton transfer process.

<table>
<thead>
<tr>
<th>Reaction Coordinate (Å)</th>
<th>SV(P) energy (a.u.)</th>
<th>Relative SV(P) energy (kcal/mol)</th>
<th>TZVPP energy (a.u.)</th>
<th>Relative TZVPP energy (kcal/mol)</th>
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**Supplementary Table 2:** Calculated bond lengths of the β-thiolactone product. Stabilization of the intermediate is achieved by stretching of the carbonyl (C-O) and C-Cα bonds.

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<th>Reaction Coordinate (Å)</th>
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<th>Cβ-Sγ</th>
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Supplementary Figure 1: Depiction of atoms included in the QM layer of the hybrid QM/MM simulations of the mechanism of hydrolysis by E. coli YbaK. A total of 128 atoms were included in the QM layer and consisted of the ribose moiety of terminal A76, phosphate of C75, substrate cysteine, side chains of N28, S129, and D136 residues, backbone atoms of N28-G30 and L99-S104 residues, and three structural waters.
Supplementary Figure 2: Structural alignment of YbaK and ProRS-INS domain. *E. coli* YbaK (cornflower blue) and *E. faecalis* ProRS-INS domain (green) share very high structural similarity, with RMSD of 2.0 Å (left). The Ile263 residue of the INS domain, which contributes to alanine substrate-specificity, aligns with the Gly30 residue of *E. coli* YbaK (right). The loop containing this residue (highlighted in red) is disordered in the structure of *H. influenzae* YbaK (left, gray structure). The location of the disordered loop is indicated by the red dashed line.
Supplementary Figure 3: Sequence alignment of YbaK subfamily.

A strictly conserved and functionally important Lys residue is marked by a hash (#).
Supplementary Figure 4: Deacylation of Cys-tRNA$_{Pro}$ by YbaK mutants. G30V and G30I YbaK variants do not show Cys-tRNA$_{Pro}$ deacylation activity at 0.5 µM enzyme concentration (see Figure 2A of main text), but weakly deacylate this substrate at higher enzyme concentrations (3 µM), suggesting that these mutations result in binding defects.
Supplementary Figure 5: Bond-formation in the first step of YbaK-catalyzed Cys-deacylation. The S-C bond formation during substrate cysteine cyclization corresponds to the highest energy barrier during catalysis.