Note

Pyrrolysine Analogs as Substrates for Bacterial Pyrrolysyl-tRNA Synthetase in Vitro and in Vivo

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Pyrrolysine-tRNA\textsuperscript{Pyl} complex is produced by pyrrolysyl-tRNA synthetase (PylRS). In this study, we investigated the substrate specificity of Desulfitobacterium hafnience PylRS. PylRS incorporated various l-lysine derivatives into tRNA\textsuperscript{Pyl} in vitro. In addition, the PylRS/tRNA\textsuperscript{Pyl} pair introduced these lysine derivatives into the recombinant protein by the Escherichia coli expression system, indicating that this PylRS/tRNA\textsuperscript{Pyl} pair can be used in protein engineering technology.

Key words: Desulfitobacterium hafnience; pyrrolysine; pyrrolysyl-tRNA synthetase

Pyrrolysine (Pyl) was the twenty-second natural amino acid discovered in 2002. It was found to be present in the mono-, di-, and tri-methylamine methyl-transferases of the methanogenic archaea Methanosarcina barkeri.\textsuperscript{1,2} Pyl is biosynthesized from two lysine (Lys) molecules by three biosynthesis enzymes, PyIB, PyIIC, and PyILD.\textsuperscript{3} It is then introduced to tRNA, which corresponds to the UAG amber codon by pyrrolysyl-tRNA synthetase (PylRS), and is incorporated into the proteins in the in-vitro protein expression system.\textsuperscript{5–10} PylRS is well characterized. The crystal structure of PylRS indicates that this enzyme possesses a wide substrate binding pocket larger than that of M. barkeri PylRS.\textsuperscript{11,12} However, unlike M. barkeri PylRS, the substrate specificity of DhPylRS has not yet been well investigated, and only a few studies have been reported.\textsuperscript{13}

Recent protein engineering efforts make it possible to add non-canonical amino acids to the genetic code.\textsuperscript{13} In this technology, an orthogonal aminoacyl tRNA synthetase (aaRS)-tRNA pair, in which aaRS specifically and exclusively acylates the orthogonal tRNA with a non-canonical amino acid, is required. M. barkeri PylRS and tRNA\textsuperscript{Pyl} have shown to function as an orthogonal pair in Escherichia coli cells.\textsuperscript{14,15} By the use of this pair, various Lys derivatives were incorporated into recombinant proteins, and the pair was applied to the site-specific modification of recombinant proteins in which a non-canonical amino acid was used as a target functional group.\textsuperscript{5–10} On the other hand, although it has been found that DhPylRS functions in E. coli cells with D. hafnience tRNA\textsuperscript{Pyl} as an orthogonal pair,\textsuperscript{12} and is presumed to recognize wide variety of Lys derivatives as substrates, similarly to the archaeal PylRS, no direct evidence as to which various Lys derivatives are incorporated into recombinant proteins by the E. coli expression system has been found. In the present study, we investigated the substrate specificity of bacterial DhPylRS in vitro and the orthogonality of the DhPylRS/tRNA\textsuperscript{Pyl} in E. coli expression system.

It has been found that the carbonyl group attached to the side-chain amino group of lysine is required for recognition by archaeal PylRS, and that Lys(Cyc) acts as a substrate of PylRS compatible with Pyl.\textsuperscript{5} As compared to archaeal PylRS, the amino acid residues associated with the substrate side-chain carbonyl group are conserved in DhPylRS, suggesting that DhPylRS also requires a carbonyl group at the side-chain amino group for substrate recognition.\textsuperscript{11,12,13} Consistently with this, it has been demonstrated that DhPylRS also recognizes Lys(Cyc) as a substrate.\textsuperscript{12} Based on these observations,
we prepared various L-lysine derivatives, as shown in Fig. 1. L-Lysine, Lys(Boc) (3), Lys(Ac) (4), Lys(Z) (5), Lys(Fmoc) (10), Lys(Aloc) (11), and Lys(Biot) (12) were purchased from commercial sources. The other Lys derivatives were chemically synthesized.

In order to determine the substrate specificity of DhPylRS, an aminoacylation assay was performed. Recombinant DhPylRS and tRNA\textsubscript{Pyl}, prepared by a previously described method, were incubated with various lysine derivatives (10 mM) in 100 mM Hepes buffer (pH 7.2) containing 2 mM dithiothreitol (DTT), 2 mM ATP, and 10 mM MgCl\textsubscript{2}. After the reaction at 37°C for 5 h, the whole tRNA was subjected to acidic polyacrylamide gel electrophoresis assay, and the gel was stained with methylene blue. The results were shown in Fig. 2. Lys(Cyc) (1) was used as substrate as positive control. In this reaction, about half of the tRNA was aminoacylated and a band derived from the Lys(Cyc)-tRNA complex was observed above the non-acylated tRNA. Similarly to the case of Lys(Cyc) (1), Lys(Proc) (8), Lys(Aloc) (11), and Lys(Meoc) (15) were well recognized by DhPylRS and the aminoacylated tRNAs were observed as thick bands on the gel. Lys(Aloc) (11) gave the thickest band of the all substrates tested, and the band derived from non-acylated tRNA was detected as only a faint band. Aminoacylated tRNAs were also found when Lys(Boc)
Substrate Specificity of Pyrrolysyl-tRNA Synthetase

(3), Lys(Troc) (6), and Lys(Mpa) (14) were used as substrates as faint bands on the gel, indicating that these lysine derivatives acted as DhPylRS substrates, but were less effective than Lys(Cyc) (1). On the other hand, no acylated-tRNA was observed on the gel when Lys(CycNH) (2), Lys(Ac) (4), Lys(Z) (5), Lys(Azoc) (7), Lys(-Pro) (9), Lys(Fmoc) (10), Lys(Biot) (12), Lys(Me, Boc) (13), or Lys(Mca) (16) was used as substrate at a concentration of 10 mM. It has been reported that Lys(Ac) (4) is a minimal structure of the archael PylRS substrate, and a higher concentration (100 mM) on aminoaacylation assay was required to detect the aminoaacylated product. To check the minimal substrate structure of DhPylRS, we examined the aminoaacylation assay using Lys(Ac) (4) at a concentration of 100 mM. A faint band derived from the Lys(Ac)-tRNA complex was observed on the gel, indicating that DhPylRS also recognized Lys(Ac) (4) as substrate at a high concentration in vitro (data not shown). DhPylRS did not esterify the tRNA when t-lysine was used as substrate, indicating that DhPylRS specifically recognized N-acylated lysine derivatives.

The results of the aminoaacylation assay clearly indicated that DhPylRS showed a substrate specificity similar to M. barkeri PylRS. A small hydrophobic group attached at the end of lysine side chain via amido bond is preferred in vitro. This is consistent with the fact that the tertiary structure of DhPylRS is similar to that of M. barkeri PylRS.

To determine the usefulness of DhPylRS in the protein engineering technology, which incorporates non-canonical amino acids into recombinant proteins, we constructed an expression system of a model recombinant protein. The D. hafniae tRNA Pyl gene was inserted into the pUC18 plasmid as described previously. Using the DhPylRS/tRNA pair to site-specific modification by click chemistry, in which alkyne is allowed to react with an azido group, forming a triazole structure. This reaction has been used for such purposes by means of the archael PylRS/tRNA orthogonal pair. Our bacterial pair might come to be an alternative pair for protein engineering technology.

In conclusion, we investigated the substrate specificity of DhPylRS in vitro and in vivo. The results indicated that it preferred lysine derivatives with a small hydrophobic acyl group attached to the side-chain amino group. This characteristic is similar to archael PylRS and this is consistent with the tertiary structure similarity. Using the DhPylRS/tRNA pair, the alkyn-containing lysine derivative Lys(Proc) was incorporated into the recombinant protein in vivo, indicating that the technique described here can be used for site-specific modification by click chemistry. Hence, DhPylRS and the tRNA Pyl of D. hafniae might be an alternative pair to the archael one in technology that makes it possible to introduce a non-canonical amino acid into recombinant proteins and to modify them site-specifically.

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References