Aminoacylation of tRNA is an essential stage during translation process. In this stage, a set of enzymes designated as aminoacyl-tRNA synthetases (aaRS) specifically match amino acids to their cognate tRNAs. In majority of organisms, CysRS catalyzes attachment of cysteine to tRNA<sup>Cys</sup> to synthesize Cys-tRNA<sup>Cys</sup>. However, CysRS and/or cysteine biosynthesis enzymes are absent in many methanogenic archaea, Cys-tRNA<sup>Cys</sup> is formed by a two-step indirect pathway in such organisms. Recent research of our group discovered that a large complex named transsulfursome takes charge of Cys-tRNA<sup>Cys</sup> synthesis in indirect pathway. Transsulfursome (540 kDa) consists of three protein components in a ratio of 4: 4: 4, O-phosphoseryl-tRNA synthetase (SepRS), Sep-tRNA:Cys-tRNA synthase (SepCysS), and SepRS/SepCysS pathway enhancer, SepCysE which is known as a bridge to connect SepRS and SepCysS. Synthesis of Cys-tRNA<sup>Cys</sup> is performed on transsulfursome by two steps: firstly O-phosphoserine (Sep) is ligated to tRNA<sup>Cys</sup> by SepRS; and then the mischarged intermediate Sep-tRNA<sup>Cys</sup> is transferred to SepCysS where it is converted into Cys-tRNA<sup>Cys</sup>. This indirect pathway is the sole source of cysteine biosynthesis in methanogenic archaea, and may be a ancestor pathway that the first time introduced cysteine into the genetic code.

Previous research revealed interaction between SepRS and tRNA<sup>Cys</sup> in the first step of indirect pathway; in contrast, interaction between SepCysS and tRNA<sup>Cys</sup> in the second step remains unclear. In this study, I determined structure of SepCysS-SepCycE with tRNA<sup>Cys</sup>, which represents a binding manner of tRNA<sup>Cys</sup> in the second step. The structure of SepCysS-SepCysE-tRNA<sup>Cys</sup> consists of a SepCysS dimer, a SepCysE dimer and only one tRNA<sup>Cys</sup>, leaving one active site empty. Interestingly, in this complex, tRNA-bound SepCysE contains two domain; SepCysS-binding domain (CSBD), and tRNA-binding (tRBD), while tRBD disordered in tRNA-free one. Three regions of SepCysS-SepCysE interact with tRNA<sup>Cys</sup>. The N-terminal loop (L16-L30) of SepCysS crosses over the ΨC loop of tRNA<sup>Cys</sup>, and this weak
interaction is considered important to direct acceptor arm into active site. In addition, a helix-loop-helix-loop region (P333-E367) of SepCysS at the entrance of active site compactly interacts with acceptor arm of tRNA, in which four residues (R345, G346, F347, G364) specifically recognize the discriminator base U73. Binding assay showed that mutant of tRNA\textsuperscript{Cys} U73A lost binding ability to SepCysS-SepCysE, while substitution of G73 with uracil in tRNA\textsuperscript{Phe} acquired binding ability to the complex. As previous research already revealed the recognition of tRNA\textsuperscript{Cys} at anticodon and U73 by SepRS in the first step, recognition of tRNA\textsuperscript{Cys} at U73 by SepCysS suggests that the two-step process is rigorously scheduled on transsulfursome to guarantee fidelity of the reactions. Moreover, in third tRNA interaction region tRBD, the protein-tRNA interface revealed a positive charged region of tRBD to interact with the elbow of tRNA\textsuperscript{Cys} via electrostatic interaction. Further investigation on binding affinity of SepCysE(tRBD) and tRNAs by EMSA indicates that tRBD is a non-specific tRNA binding domain, as it can bind different tRNAs with affinity almost at the same level as tRNA\textsuperscript{Cys}.

In order to understand how transsulfursome assembles and tRNA channeling on it, I obtained a structure of SepRS and SepRS-bound N-terminal helix of SepCysE (named RSBD: SepRS-binding domain), although the full transsulfursome mixed with tRNA\textsuperscript{Cys} was used to crystallize, as the rest parts of transsulfursome disorder in the structure. The packing of structure shows there are enough spaces at the two sides (the side of C-terminus of N-helix) of RSBD to accommodate the other parts of SepCysS-SepCysE. In this structure, two dimers of RSBD insert deeply into clefts at interfaces of anticodon binding domains of SepRS tetramer (dimer of dimer). Three loops of SepRS at the interface are mainly responsible for binding RSBD. These interactions were confirmed by binding assay of SepRS and SepCysE mutants. Truncation of each loop of SepRS separately showed no significant decrease on binding affinity, while complex formation of SepRS and SepCysE cannot be observed when deleting RSBD or introducing double-loop truncations of SepRS. This tight binding manner facilitates the formation of transsulfursome. Furthermore, prediction of secondary structure of SepCysE suggests that the RSBD is followed by a long loop, which is invisible in current structures and explains the flexibility of SepCysS-SepCysE. Taken current structures and assay data together, it can be concluded that SepCysE consists of three domains, RSBD (M1-I25; N-terminal helix), CSBD (P41-F98; two helices), and tRBD (L112-D213; C-terminal domain), connected by two long loops, linker1 (R26-K40) and linker2 (G99-A111).

Furthermore, a model of transsulfursome without SepCysE(tRBD) was built by superposing 2-fold axes of SepRS-SepCysE(RSBD) and SepCysS-SepCysE(CSBD) structures. This transsulfursome model was further supported by SEC-SAXS and TEM data, which also indicated the flexibility of two flanking SepCyS-SepCysE. Finally, models of transsulfursome-tRNA in the first and the second step were also built by superposing tRNAs of SepCysS-SepCysE-tRNA and SepRS-tRNA. Taken all together, I proposed the tRNA channeling on transsulfursome via cooperation work of two linkers of SepCysE.

In conclusion, in this study, the applicant has new findings on the process of Cys-tRNA\textsuperscript{Cys} synthesis in indirect pathway by transsulfursome, and these findings will contribute important information to life science (especially in the field of tRNA research).

Therefore, we acknowledge that the applicant is qualified to be granted the Doctorate of Life Science from Hokkaido University.