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SUMMARY OF DOCTORAL THESIS

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Title: **Mutagenesis studies of L-carnitine dehydrogenase for development of biomolecule measurement tool**

（生体分子測定ツールの創製に向けたカルニチン脱水素酵素のタンパク質工学的研究）

Naturally occurring quaternary ammonium compound L-carnitine (L-Car), as an essential biological nutrient, has been well investigated. Thereafter, several approaches have been established for measurement the concentration of L-Car in the clinical diagnosis and nutrient analysis. Of them, a spectrophotometric assay method using L-carnitine dehydrogenase (CDH) has been proposed as a superior tool (easy, rapid and low-cost) for L-Car measurement. While several CDHs have been purified and characterized from different soil isolate bacteria, no study has been conducted in the structure function relationship for any of CDHs. Therefore, the current study was conducted to investigate the role of several amino acid residues in the catalytic activity and substrate affinity that could provide new and useful information insight into the catalytic mechanism for improvement the properties of two recombinant CDHs isolated from *Xanthomonas translucens* (Xt-CDH) and *Rhizobium* sp. (Rs-CDH).

Chapter 1 outlines the rationale and aims of the current dissertation, summarizes relative studies of L-Car properties, *in vivo* biosynthesis, biological functions, deficiency and assessment and also provides a brief note on CDH research progress.

In Chapter 2, the study was conducted to identify residues important for affinity to the substrate of CDHs. Based on the substrate affinity variation of Xt-CDH (10 mM) and Rs-CDH (1 mM), we compared the primary structure of Xt-CDH and Rs-CDH with the recognized 3D structure of human 3-hydroxyacyl-CoA dehydrogenase (h-HAD) (PDB code: 1F0Y). In the h-HAD structure, several residues surround the acetoacetyl moiety of the substrate namely the residues Lue157, Phe160, Gly204, Ile206, Val207, Gly239, and Ala240. These residues matched Gly140, Phe143, Gly188, Ile190, Ala191, Gly223, and Ala224 of Xt-CDH, which correspond to Ala137, Tyr140, Ala185, Val187, Gly188, Ser220, and Phe221 in Rs-CDH. The residues of Xt-CDH were replaced with that of Rs-CDH at the corresponding position and *vice versa*. The resulting mutant enzymes were purified and subjected to kinetic characterization. All Rs-CDH mutants exhibited slight effects on substrate affinity, except for the double mutants Rs-V187I/G188A, which was devoid of enzyme activity. For Xt-CDH mutants,

Xt-F143Y, Xt-I190V/A191G, and Xt-G223S/A224F significantly elevated the K_m value, implicating the residues in L-Car binding. Of them, mutation of Phe143 with Tyr caused a higher increase in the K_m value. Therefore, mutant Xt-F143Y and its corresponding in Rs-CDH (Rs-Y140F) were further characterized. The preferred pH of Xt-F143Y was shifted from 9.5 to 8.0 compared to that of corresponding mutant (Rs-Y140F). Moreover, the kinetic parameters of additional mutants at Xt-F143 and Rs-Y140 (replaced with residues Ala, Gly, Lys, Cys, Ser, Asn, His, Asp, Trp, and Phe) were scrutinized. All Rs-Y140 mutants, except aromatic residues (Rs-Y140F and Rs-Y140W), produced proteins that were almost entirely devoid of enzyme activity and with disrupted affinity to L-Car. Additionally, all Xt-F143 variants, event that of Xt-F143Y and Xt-F143W, exhibit consistent reduction in enzyme activity and substrate affinity. Analysis of these mutants provides a better understanding of the structural and functional relationships between CDHs and its ligand L-Car. The effects of residues Xt-F143 and Rs-Y140 on catalytic activity and substrate affinity were unforeseen. As the aromatic property of these residues does not the sole factor, results emphasize the important role for physical properties, such as volume, of residues might play essential role in substrate recognition.

The study in Chapter 3 was carried out to explore functionally important resid ues in Xt-CDH. A total of 42 residues were selected for site-directed mutagenesis based on their strict homology among the CDHs and bacterial 3-hydroxyacyl-Co A dehydrogenase. All residues were substituted with alanine (alanine scanning mut agenesis). The resultant mutants were analyzed for catalytic activity. Five of the mutant enzymes were inactive (E93A, H141A, E153A, D217A, and R227A). Acti ve mutants were evaluated for their influence on L-Car affinity using assay mixtu res of different L-Car concentration (12, 300, and 900 mM). Based on the obtaine d results, mutants could be categorized into three groups; mutants influenced k_{cat} only (R136A, D192A, R221A, and Q284A), mutants altered k_{cat} and K_m for L-Car (S117A, S120A, N144A, Y147A, E185A, F189A, R193A, E196A, W199A, R200 A, E201A, W228A, M231A, F234A, Y237, M246A, F249A, Q252A, F253A, W2 61A, T262A, R297, and D298A), and mutants had similar approach to that of Xt -CDH (T119A, H133A, M177A, K184A, T236A, R247A, H248A, S289A, E294A, and R295A). The mutant enzymes affected the K_m values were subjected for ste ady kinetics analysis. The analytical data implied that all mutants had increased K_m value. Of those, R193A, E196A, W199A, R200A, F249A, and F253A that prod uced the greatest L-Car affinity disruption (K_m >200-folds of Xt-CDH) clustered n ear the putative active site. Overall, the reported data of this section underscores t he important conserved residues for substrate affinity of the entire catalytic domai n, which could be considered as blueprint for the rational of further intensive mut agenesis studies to improve the catalytic function and substrate affinity of CDH.