CHAPTER IV

DISCUSSION

4.1 Purification of L-alanine dehydrogenase from *Aeromonas hydrophila*

Aeromonas hydrophila was screened from Bangkok soil by Phungsangthum (1997). It produces alanine dehydrogenase, which is known as one of the important enzymes in the assimilation of L-alanine as an energy source through the TCA cycle in several organisms (McCowen and Phibbs, 1974; O' Connor and Halvoson, 1960). The physiological function of alanine dehydrogenase from *Aeromonas hydrophila* has not been reported. However, the enzyme from other sources such as *Bacillus* sp. (Ohshima and Soda, 1979; Yoshida and Freese, 1964; Muresan *et al.*, 1983), were found in the vegetative cells and sporulation stage. In N_2- fixing bacteria, alanine dehydrogenases have an important role in nitrogen assimilation. Although glutamine synthetase/ glutamate synthase (GS/GOGAT) are more important than alanine dehydrogenase in the primary NH_4^+ assimilation, alanine dehydrogenase may become more important when the availability of nitrogen increases during the period of the nodule development (Sawa *et al.*, 1994; Smith and Emerich, 1993).

The alanine dehydrogenase from *Aeromonas hydrophila* has been purified and characterized by Phungsangthum (1997). In this work, the purified enzyme was obtained after 4 steps of purification: 30-40% ammonium sulfate precipitation, DEAE-Toyopearl, hydroxyapatite and Blue Sepharose column chromatography. The fifth step of Sephadex G-200 column used in Phungsangthum's study was omitted in this study. When the data of purification was analyzed, the enzyme was purified to 150.8 folds and the specific activity was 18.09 units/mg protein with 24.8 % yield. The result was similar to Phungsangthum who demonstrated that the enzyme was 100 folds purified with the specific activity of 23.0 units/mg protein and 18.4 % yield.

4.2 Identification of amino acid residues in the active site of alanine dehydrogenase

Several methods for investigation of amino acid residues which are essential for function or structure of protein such as affinity labelling with their substrate or substrate analogue (Hirano *et al.*, 1991), X-ray crystralography (Baker *et al.*, 1998), site directed mutagenesis (Boehlein *et al.*, 1997) or chemical modification (Bhattacharyya *et al.*, 1997) have been reported.

Chemical modification is one of the most useful method of identifying the functional groups of a protein. Whether or not the various types of amino acid side-chains are involved in a protein function can be determined readily by whether or not chemical modification of that type of amino acid affects the function. This requires only chemical reactions that are reasonably specific for each type of amino acid side-chain (Appendix D). Suitable specific reactions are available for aspartic acid, glutamic acid, lysine, arginine, methionine, tryptophan, tyrosine and cysteine residues. A few, less satisfactory, reactions are available for serine, threonine, asparagine and glutamine residues, but none are available for glycine, alanine, valine, leucine, isoleucine and proline, which have no reactive groups on their side-chains. However, reactive groups are usually required for important functional roles, so chemical modification is able to test all the likely functional amino acids.

Having determined which type of amino acid side-chains are involved, it is also desireable to identify which specific residues of that type in a protein are responsible. This requires that the different residues be distinguished by their reactivities. Fortunately, the residues involved in protein function often have unusual reactivities or environments that make them distinguishable in this way (Imoto and Yamada, 1989).

Inactivation of enzyme with or without its substrate may suggest that the inactivation is due to a direct modification of essential amino acid residues at the enzyme active site or due to the modification of amino acid residues remote from the active site

(Dong *et al.*, 1991). The recently developed technology of gene engineering using a site-directed mutagenesis has an advantage of altering protein structure. However, this approach is limited by the need of the information about the residues of the protein, which are likely to be involved in active site. The chemical modification can provide just this information. Thus, the two techniques are complementary, chemical modification can identify the residues likely to be involved, and gene engineering can be elucidated in detail their roles in the biological function.

In this work, the investigation of amino acid residues in the active site for alanine dehydrogenase from *Aeromonas hydrophilla* was performed by chemical modification with group-specific modifying reagents. Chemical modification of six amino acid residues, tyrosine, methionine, histidine, cysteine, arginine and lysine were tested under mild condition.

The first step was screening of the amino acid residues, of which their modification had effects on the enzyme activity. Preincubation of the purified enzyme with modifying agents resulted in variable changes in the catalytic ability of this enzyme. Methionine, histidine, arginine, and lysine which reacted with the specific reagents, chloramine T (CT), diethylpyrocarbonate (DEPC), phenylglyoxal (PG), and 2,4,6- trinitrobenzenesulfonic acid (TNBS), respectively, under mild condition, resulted in extensive inhibition of alanine dehydrogenase activity, while modification of tyrosine and cysteine did not affect the activity (Table 4). It may be assumed that arginine, histidine, lysine and methionine are all likely involved in alanine dehydrogenase activity. Comparison of the deduced amino acid sequences of the enzyme from *Aeromonas hydrophila* with linear alignment of amino acid sequence of the enzyme from other sources (Figure 6) showed that all of these amino acid residues were conserved. Kuroda *et al.*,(1990) compared the amino acid sequence of alanine dehydrogenase from *B. sphaericus* and *B. stearothermophilus* with other amino acid dehydrogenases from the protein data bank. It led them to propose that histidine-153 and lysine-126 were the part of the active site of alanine dehydrogenases from both

Bacillus. However, Andersen, *et al*., (1992) found that the result of sequence from alanine dehydrogenase of *Mycobacterium tuberculosis* did not support this hypothesis. Delforge, *et al*., (1997) reported lysine-74 was the amino acid at the pyruvate binding site of the enzyme from *B. subtilis*. Moreover, Baker, *et al*, (1998) reported that methionine, histidine, arginine and lysine played an important role for alanine dehydrogenase activity in *P. lapideum*. In contrast with CT, DEPC, PG, and TNBS, modification by DTT at cysteine residue(s) of alanine dehydrogenase showed no effect on the enzyme activity. Alanine dehydrogenase from *Aeromonas hydrophila* consists of four cysteine residues in each subunit (Poomipark, 2000). Comparison of amino acid sequence of *Aeromonas hydrophila* alanine dehydrogenase with other alanine dehydrogenases resulted in the finding that no cysteine was conserved among alanine dehydrogenases. Thus, it can be clearly concluded that cysteine residues for alanine dehydrogenase from *Aeromonas hydrophila* do not involve in the enzyme function.

For tyrosine, 89% of the residual activity was remained when the enzyme was incubated with NAI indicating that tyrosine may not be necessary for enzyme activity. Baker *et al*., performed three dimensional structure of alanine dehydrogenase from *P. lapideum* and proposed that the methyl group of pyruvate packed against the hydrophobic residues of tyr-93, met-132 and leu-129. However, the sequence obtained from *M. tuberculosis* ruled out this hypothesis since the proposed tyr-93 residue was not conserved but was substitued by phenylalanine.

The second step was to prove that the amino acid residues involved in alanine dehydrogenase activity were at the active site or not because chemical modifications did not directly indicate that the specific residues were present at the active site. If chemical modification of amino acid is protected by substrates, the loss of activity in the presence of protective substrate from inhibition should be less than in the absence, then that residue is in the active site of the enzyme. In this work, we tested the protective effect of pyruvate and/or NADH on the modification at methionine, histidine, arginine and lysine residues.

The result of enzyme inactivation by group-specific reagents in the presence or absence of protective substrate(s) shown in Table 5-8 indicated histidine, arginine, and lysine should be essential residues in an active site, which corresponded with previous reports. According to Grimshaw *et al.*, (1981), a cationic acid group on the enzyme (probably a lysine) is required for effective binding of the substrates and inhibitors while another cationic group (probably a histidine) acts as an acid-base catalyst of the reaction. Moreover Baker *et al.*, (1998), proposed that in addition to interaction of methyl group of pyruvate to hydrophobic residues, carboxyl hydrogen of pyruvate was bonded to the side chain of arg-15. The side chain amino group of lys-74 interacts with both the carbonyl oxygen of the pyruvate and one of its carboxyl oxygens. A hydrogen bond is also made between the carbonyl oxygen of the pyruvate and the side chain of his-95 and N δ from the side chain of asn–229 interacts with one of the carboxylate oxygens of the pyruvate. The chemical mechanism was proposed to proceed through iminopyruvate and carbinolamine intermediates in which lys-74, arg-15 and his-95 were catalytic residues (Appendix G).

The third step of chemical modification experiment was to study kinetics of inactivation of histidine and arginine residues for estimation of the pattern and the stoichiometric reaction ratio of chemical reagent to monomer of enzyme. The enzyme was incubated with varying concentrations of DEPC or PG for modification of histidine or arginine, respectively. In both cases, the inactivation of the enzyme was a result of a simple bimolecular reaction, the plotting of log k_{inact} versus log of chemical concentration gave a slope value of 1.0-1.1, which indicated that the inactivation resulted from the reaction of approximately 1 mole of reagent with 1 mole of enzyme subunit (Figure 20 and 21).

The fourth step was to determine the pattern of substrate protection. The ability of substrate to protect the amino acid at the active site of the enzyme from inactivation by DEPC or PG at various time of incubation was measured (Figure 22 and 23). The mechanism of reaction of alanine dehydrogenase from *Aeromonas hydrophila* is known to be the sequential ordered binary-ternary mechanism when NADH binds before pyruvate.

We tested the protection of the enzyme active site in four conditions and found that the K_{inact} was reduced when NADH and pyruvate were used together. This result clearly showed that the histidine or arginine residues reacted with these modifying reagents were located at the active site of the enzyme and could only be protected in the presence of the ternary complex E•NADH•pyruvate. The inability of NADH alone to protect the active site of the enzyme, because the modification did not occur at the NADH binding site itself but more likely at or near the pyruvate binding site of the enzyme as the presence of the pyruvate is also required for good protection. On the other hand, according to the kinetic mechanism of this enzyme, puruvate can bind with the binding site after NADH is already fixed to the active site (Phungsangthum, 1997). Thus, it is possible that modified histidine and arginine are at the pyruvate binding site.

The final step was to localize histidine residue(s) in the active site of alanine dehydrogenase. Alanine dehydrogenase was modified by DEPC and digested with lysylendopeptidase, then the peptides were separated by HPLC. The profiles of digested peptides of native and DEPC-modified enzymes were different (Figure 24). Peptide maping of native enzyme and DEPC-modified enzyme allowed the identification of the active site peptide fragments. Sequence comparisons of the isolated fragments with known sequences of alanine dehydrogenases from various sources including *Aeromonas hydrophila* indicated that his-95 from peak I and his-31 from peak III might be essential residues. The his-12 from peak III is not conserved among alanine dehydrogenases, therefore, it should be ruled out. Since the ratio of DEPC: enzyme monomer is 1:1, only one histidine should play a role in an active site. Three dimentional structure of alanine dehydrogenase from *P. lapideum* showed that his –31 locates at the surface of the enzyme subunit wherease his-95 was in the active cleft with eight conserved residue LFTYLHLA from leu-90 to ala-97. However tyr-93 is replaced by phenylalanine in *M. tuberculosis*, which suggests the non conserved position at residue 93. Thus, his-95 of alanine dehydrogenase from *Aeromonas hydrophila* may also be essential amino acid residue in pyruvate binding site. HPLC profile of peptide fragments from substrates protection should be performed to confirm the active site location of these histidine residues.

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   1 ATGATTATCGGTGTACCTAAGGAAATCAAAAACCACGAATATCGCGTAGGCATGGTTCCG
     M  I  I  G  V  P  K  E  I  K  N  H  E  Y  R  V  G  M  V  P
  61 GCCAGTGTACGTGAACTGACAGCACGAAACCATACCGTTTTCGTCCAAAGCGGCGCAGGA
     A  S  V  R  E  L  T  A  R  N  H  T  V  F  V  Q  S  G  A  G
 121 AATGGCATTGGGTTCAGTGACGCAGATTATCTGGCTGCCGGAGCCGAGATCCTGGCCTCT
     N  G  I  G  F  S  D  A  D  Y  L  A  A  G  A  E  I  L  A  S
 181 GCGGCAGACGTTTTCGCCAAGGCGGAGATGATCGTCAAGGTCAAGGAGCCCCAGGCGGTC
     A  A  D  V  F  A  K  A  E  M  I  V  K  V  K  E  P  Q  A  V
 241 GAGCGCGCCATGCTGCTGCCGGGCACGACCCTCTTTACCTACCTGCACCTGGCGCCAGAC
     E  R  A  M  L  R  P  G  Q  T  L  F  T  Y  L  H  L  A  P  D
 301 CTGGCCCAGACCCGGGAGCTGGTGGACAGCGGCGCTATCTGAATCGCCTACAAAACCGTC
     L  A  Q  T  R  E  L  V  D  S  G  A  I  C  I  A  Y  K  T  V
 361 ACCGACGGCCGTGGCGGCCTGCCCCTGCTGGCCCCCATGTCGGAGGTGGCCGGACGCATG
     T  D  G  R  G  G  L  P  L  L  A  P  M  S  E  V  A  G  R  M
 421 TCTATTCAGGCGGGTGCCCAGGCGCTGGAAAAATCCCGCGGCGGTAGCGGCGTGCTGCTC
     S  I  Q  A  G  A  Q  A  L  E  K  S  R  G  G  S  G  V  L  L
 481 GGCGGCGTGCCCGGCGTGGAACCGGCCAAGGTGGTGATCATCGGCGGCGGCGTGGTGGGC
     G  G  V  P  G  V  E  P  A  K  V  V  I  I  G  G  G  V  V  G
 541 TCCAACGCAGCCCGCATGGCCATCGGCCTGCGTGCCGACGTCACCATACTCGACAACAAC
     S  N  A  A  R  M  A  I  G  L  R  A  D  V  T  I  L  D  N  N
 601 ATCGATACCCTGCGCCGTCTCGACAGCGAGTTCCAGGGTGCCGCCAAGGTGGTTTACTCC
     I  D  T  L  R  R  L  D  S  E  F  Q  G  A  A  K  V  V  Y  S
 661 AACCGCGAGACCCTGGAGCGCCATTTGCTGGCGGCAGACCTGGTTATCGGTGGCGTGCTG
     N  R  E  T  L  E  R  H  L  L  A  A  D  L  V  I  G  G  V  L
 721 GTACCGGGCGCCACTGCGCCCAAACTTGTCAGCCGTGACCACATTGCCCGCATGAAGCCG
     V  P  G  A  T  A  P  K  L  V  S  R  D  H  I  A  R  M  K  P
 781 GGGTCGGCCATTGTGGACGTGGCCATCGATCAGGGCGGCTGCGTCGAGACCTCCCATGCC
     G  S  A  I  V  D  V  A  I  D  Q  G  G  C  V  E  T  S  H  A
 841 ACCACCCATGAGGATCCCACCTTCATTGTCGACGACGTGGTGCACTACTGCGTGGCCAAC
     T  T  H  E  D  P  T  F  I  V  D  D  V  V  H  Y  C  V  A  N
 901 ATGCCGGGCGCCGTGGCACGCACCTCCACCGTGGCCCTGAACAACGCCACCGTGCCCTTC
     M  P  G  A  V  A  R  T  S  T  V  A  L  N  N  A  T  L  P  F
 961 ATCATCAAGCTGGCCGAACAGGGCTATCGCAACGCACTGCTCAGCGATCCCCACCTGCGG
     I  I  K  L  A  E  Q  G  Y  R  N  A  L  L  S  D  P  H  L  R
1021 CACGGCCTGAACGTGATGGCGGGCAAAATCACCTGCAAGGAGGTCGCCGTGGCCCACAAC
     H  G  L  N  V  M  A  G  K  I  T  C  K  E  V  A  V  A  H  N
1081 CTGGCCTACACGGATCCCCTGACCCTGCTGAACTGATCGCATCCCCGCGAGGGGAGGTAG
     L  A  Y  T  D  P  L  T  L  L  N  *
1141 ATGAGTGAAAATCCGCCCGCGATGGGCGGTTTTTTTTTATGCCTGCCATCCCCCCTGCC
1201 CCTCTCTTTGGCAATTGCCCCAATCAATGCGCCGGCCTTTGACCACGGATCACAAATTAC
1261 CTAAATCGCGCAAAGTGATC
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Figure 25 The nucleotide sequence and the deduced amino acid sequence of alanine dehydrogenase from *Aeromonas hydrophila*

Underlines show N-terminal amino acid sequences of two interesting peptide fragments