Molecular Mechanism of Lipase Activator Protein from *Pseudomonas aeruginosa*

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CONTENTS

ABBREVIATIONS

LipA	lipase from *Pseudomonas aeruginosa* TE3285
Δ21LipB	LipB lacking 21 residues at the N-terminal
Δ61LipB	LipB lacking 61 residues at the N-terminal
H6-Δ61LipB	Δ61LipB fusion protein containing hexahistidyl leader sequence
ATP	adenosine 5'-triphosphate
BCA	bicinchoninic acid
EDTA	ethylenediaminetetraacetic acid
MES	2-morpholinoethanesulfonic acid
PAGE	polyacrylamidegel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulfate
Tris	tris(hydroxymethyl)aminomethane

CHAPTER 1

General Introduction

Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) catalyzes hydrolysis of triacylglycerol into fatty acids and glycerol. This enzyme is distributed among a number of organisms from bacteria to mammalians. In general, lipases possess characteristic properties most enzymes do not. First, their natural substrates, long-chain fatty acyl esters, are usually insoluble. Second, lipases remain stable and active in a variety of organic solvent (Zaks and Klibanov, 1984), so that catalyze reverse reactions in the ester synthetic direction. Third, reactions catalyzed by lipases show high enantioselectivity around alcohol leaving group of substrate. For these reasons, lipases are expected as a versatile catalyst for potential applications such as food industry, detergent formulation, and medicinal chemistry. Particularly, lipases produced from Gram-negative bacterial genus *Pseudomonas* possess not only the above features, but also practical availability as follows (Jaeger *et al.*, 1994). *Pseudomonas* lipase is one of the extracellular enzymes, thus the purified sample can be easily prepared from culture medium (Stuer *et al.*, 1986, Gilbert *et al.*, 1991, Chihara-Siomi *et al.*, 1992, Sugihara *et al.*, 1992, Taipa *et al.*, 1992). In addition, enzymatic characteristics of the lipase such as thermostability (Iizumi *et al.*, 1990, Sugihara *et al.*, 1992) or substrate specificity (Inagaki *et al.*, 1989a, Ihara *et al.*, 1991) vary according to its producing species of *Pseudomonas*. Typically, LipA (molecular weight of 30,100), an extracellular lipase from *P. aeruginosa* TE3285, expresses a characteristic substrate specificity, and can be utilized for kinetic resolution to obtain optically active chemicals. Namely, LipA catalyzes the stereoselective acylation of [1,1'-binaphthyl]-2,2'-diol (binaphthol) (Inagaki *et al.*, 1989a), benzaldehyde cyanohydrin (mandelonitrile) (Inagaki *et al.*, 1989b), and α-aminonitrile (Nakai *et al.*, 1992) (Fig. 1-1). Notably, the kinetic resolution of binaphthol through acylation was accomplished only by *P. aeruginosa* LipA, but not by lipases from other organisms such as *P. cepacia* M-12-33 (Inagaki *et al.*, 1989a, Nishioka *et al.*, 1991). This result suggests that LipA possesses a substrate recognition site distinct from that of the other lipases. Therefore, it is expected that analyses of LipA based on

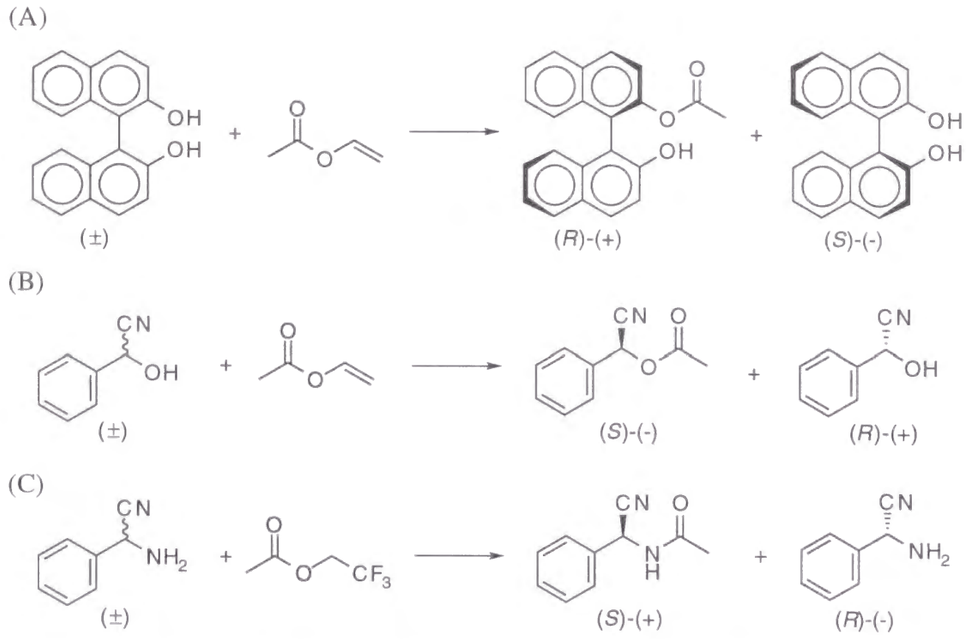

Fig. 1-1. Stereoselective acylation catalyzed by LipA, lipase from *Pseudomonas aeruginosa* TE3285. (A) Acylation of binaphthol (Inagaki *et al.*, 1989a); (B) acylation of mandelonitrile (Inagaki *et al.*, 1989b); and (C) acylation of α-aminonitrile (Nakai *et al.*, 1992).

protein structure could elucidate this substrate recognition mechanism, and to give an insight into design of a noble enzyme to catalyze a particular reaction.

For this purpose, at the beginning, the structural gene (*lipA*) of the *P. aeruginosa* LipA has been cloned and sequenced (Chihara-Siomi *et al.*, 1992) (Fig. 1-2). The *lipA* gene consists of an open reading frame of 936 bases, and encodes a polypeptide of 311 amino acid residues containing a signal sequence of 26 residues at the N-terminus. However, when only the *lipA* gene was introduced into *E. coli* host-vector system, no detectable lipase activity was observed. This observation caused by a fact that an additional DNA region at closely downstream of *lipA* was essential for the production of the active LipA protein (Chihara-Siomi *et al.*, 1992). This region includes another set of Shine-Dalgarno sequence and subsequent open reading frame (*lipB*) of 1,023 bases, which is deduced to encode 340-residue polypeptide. On the basis of the gene structure, *lipA* and *lipB* are likely to form a single bicistronic operon. These features suggest that the *lipB* gene product (LipB; molecular weight of 37,700) specifi-

cally acts on active LipA production system. However, even in the presence of *lipB*, remarkable fractions of the LipA polypeptide are produced as inactive inclusion bodies in *E. coli*. In order to establish more effective production system of LipA, it is important to understand function of LipB in molecular level.

It has been found by *in vitro* experiments that LipB assists folding of LipA into its active conformation (Oshima-Hirayama *et al.*, 1993). In this study, LipB was prepared as a recombinant fusion protein with glutathione transferase. This fusion protein recovered the lipase activity of two preparations of inactive LipA, that is, recombinant LipA solubilized from inclusion bodies accumulated in *E. coli*, and denatured LipA purified from the culture medium of *Pseudomonas*. These results suggest that LipB refolds and reactivates LipA losing the active structure at a post-translational event in the cell. This function seems to be similar to molecular chaperones, which act on a number of non-native polypeptides so as to mediate their folding at stages of biosynthesis and translocation in the cell (Ellis, 1987, Hartl, 1996). One of the common characteristics of the chaperones is that their binding of the substrate polypeptide is coupled with their ATP hydrolytic activity. On the other hand, LipB-assisted reactivation was not affected by ATP or magnesium ion (Oshima-Hirayama *et al.*, 1993). Thus, LipB is a different type of folding factor from general molecular chaperones. So far, protein folding factors similar to LipB have been found only from *Pseudomonas* species (Gilbert, 1993) and *Acinetobacter*, that is, LimL from *Pseudomonas* sp. 109 (Ihara *et al.*, 1992, Ihara *et al.*, 1995), LipH from *P. aeruginosa* PAO1 (Wohlfarth *et al.*, 1992), LimA from *P. cepacia* DSM3959 (Jørgensen *et al.*, 1991, Hobson *et al.*, 1993, Aamand *et al.*, 1994, Hobson *et al.*, 1995), Act from *Pseudomonas* sp. KWI-56 (Iizumi *et al.*, 1991, Iizumi and Fukase, 1994), LipX from *P. cepacia* M-12-33 (Nakanishi *et al.*, 1991), LipB from *P. glumae* PG1 (Frenken *et al.*, 1993a, Frenken *et al.*, 1993b), and LipB from *A. calcoaceticus* BD413 (Kok *et al.*, 1995). All of these proteins are encoded closely to the gene of the corresponding lipase. Amino acid sequences of the lipase activator proteins from *Pseudomonas* species are aligned in Fig. 1-3. LimL and LipH show more than 90% sequence identities against *P. aeruginosa* LipB, whereas the other four activator proteins show less than 30% identities. This propensity among the activator proteins is applied to that among the corresponding lipases from the respective species (Fig. 1-4). In any case, these activator proteins

CHAPTER 1

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   1 GTCGACCATTTCAGCCTGTTTTGCTCGCAAAACGACGCCGCGGGCGTGCGCACCGCACAC
  61 TCGGTCGCTGGGCGTTGTGCGGGGAAGATTCAAACGAGCGTTTCGCGCCGTAACAACCCG
 121 CCTCTTCCGCTCTGCCACGCAGGTTATGACCGGCCGCCAGGAAGCCGCGGATTTCCTGGC
 181 CTGGAGGAAAAAAGCCGAAGCTGGCACGGTTCCTGCGCAAGGGACAGCGAAGCGGTTCTC
 241 CCGGAAGGATTCGGGCGATGGCTGGCAGGACGCGCCCCTCGGCCCCATCAACCTGAGATG
 301 AGACAACATGAAGAAGAAGTCTCTGCTCCCCCTCGGCCTGGCCATCGGCCTCGCCTCTCT
-26  LipA |M  K  K  K  S  L  L  P  L  G  L  A  I  G  L  A  S  L
 361 CGCTGCCAGCCCTCTGATCCAGGCCAGCACCTACACCCAGACCAAATACCCCATCGTGCT
  -8  A  A  S  P  L  I  Q  A ▲S  T  Y  T  Q  T  K  Y  P  I  V  L
 421 GGCCCACGGCATGCTCGGCTTCGACAACATCCTCGGGGTCGACTACTGGTTCGGCATTCC
  13  A  H  G  M  L  G  F  D  N  I  L  G  V  D  Y  W  F  G  I  P
 481 CAGCGCCTTGCGCCGTGACGGTGCCCAGGTCTACGTCACCGAAGTCAGCCAGTTGGACAC
  33  S  A  L  R  R  D  G  A  Q  V  Y  V  T  E  V  S  Q  L  D  T
 541 CTCGGAAGTCCGCGGCGAGCAGTTGCTGCAACAGGTGGAGGAAATCGTCGCCCTCAGCGG
  53  S  E  V  R  G  E  Q  L  L  Q  Q  V  E  E  I  V  A  L  S  G
 601 CCAGCCCAAGGTCAACCTGATCGGCCACAGCCACGGCGGGCCGACCATCCGCTACGTCGC
  73  Q  P  K  V  N  L  I  G  H  S  H  G  G  P  T  I  R  Y  V  A
 661 CGCCGTACGTCCCGACCTGATCGCTTCCGCCACCAGCGTCGGCGCCCCGCACAAGGGTTC
  93  A  V  R  P  D  L  I  A  S  A  T  S  V  G  A  P  H  K  G  S
 721 GGACACCGCCGACTTCCTGCGCCAGATCCCACCGGGTTCGGCCGGCGAGGCAATCCTCTC
 113  D  T  A  D  F  L  R  Q  I  P  P  G  S  A  G  E  A  I  L  S
 781 CGGGCTGGTCAACAGCCTCGGCGCGCTGATCAGCTTCCTTTCCAGCGGCAGCACCGGTAC
 133  G  L  V  N  S  L  G  A  L  I  S  F  L  S  S  G  S  T  G  T
 841 GCAGAATTCACTGGGCTCGCTGGAGTCGCTGAACAGCGAGGGGGCCGCGCGCTTCAACGC
 153  Q  N  S  L  G  S  L  E  S  L  N  S  E  G  A  A  R  F  N  A
 901 CAAGTACCCGCATGGCGTCCCCACCTCGGCCTGCGGCGAGGGCGCCTACAAGGTCAACGG
 173  K  Y  P  H  G  V  P  T  S  A  C  G  E  G  A  Y  K  V  N  G
 961 CGTGAGCTATTACTCCTGGAGCGGTTCCTCGCCGCTGACCAACTTCCTCGATCCGAGCGA
 193  V  S  Y  Y  S  W  S  G  S  S  P  L  T  N  F  L  D  P  S  D
1021 CGCCTTCCTCGGCGCCTCGTCGCTGACCTTCAAGAACGGCACCGCCAACGACGGCCTGGT
 213  A  F  L  G  A  S  S  L  T  F  K  N  G  T  A  N  D  G  L  V
1081 CGGCACCTGCAGTTCGCACCTGGGCATGGTGATCCGCGACAACTACCGGATGAACCACCT
 233  G  T  C  S  S  H  L  G  M  V  I  R  D  N  Y  R  M  N  H  L
1141 GGACGAGGTGAACCAGGTCTTCGGCCTCACCAGCCTGTTCGAGACCAGCCCGGTCAGCGT
 253  D  E  V  N  Q  V  F  G  L  T  S  L  F  E  T  S  P  V  S  V
1201 CTACCGCCAGCACGCCAACCGCCTGAAGAACGCCAGCCTGTAGGACCCCGGCCGGGGCCT
 273  Y  R  Q  H  A  N  R  L  K  N  A  S  L
1261 CGGCCCCGGCCCTTTCCCGGAAGCCCCCTCGCGTGAAGAAAATCCTCCTGCTGATTCCAC
   1                    LipB |M  K  K  I  L  L  L  I  P  L
1321 TGGCGTTCGCCGCCAGCCTGGCCTGGTTCGTCTGGCTGGAACCTTCCCCCGCCCCCGAGA
  11  A  F  A  A  S  L  A  W  F  V  W  L  E  P  S  P  A  P  E  T
1381 CGGCGCCCCCGGCCAGCCCGCAGGCGGGCGCAGACCGCGCCCCGCCAGCAGCCTCCGCGG
  31  A  P  P  A  S  P  Q  A  G  A  D  R  A  P  P  A  A  S  A  G
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1441 GAGAAGCGGTGCCGGCCCCCCAGGTCATGCCGGCCAAGGTCGCGCCGCTGCCAACCTCCT
  51  E  A  V  P  A  P  Q  V  M  P  A  K  V  A  P  L  P  T  S  F
1501 TCAGGGGCACCAGCGTCGATGGCAGTTTCAGTGTCGACGCCAGCGGCAACCTGCTGATCA
  71  R  G  T  S  V  D  G  S  F  S  V  D  A  S  G  N  L  L  I  T
1561 CCCGCGACATCCGCAACCTGTTCGACTACTTCCTCAGCGCCGTCGGCGAAGAGCCCCTGC
  91  R  D  I  R  N  L  F  D  Y  F  L  S  A  V  G  E  E  P  L  Q
1621 AGCAAAGCCTGGACCGCCTGCGCGCCTACATCGCCGCCGAACTCCAGGAGCCGGCGCGCG
 111  Q  S  L  D  R  L  R  A  Y  I  A  A  E  L  Q  E  P  A  R  G
1681 GCCAGGCGTTGGCGCTGATGCAGCAATACATCGACTACAAGAAGGAACTGGTGCTGCTCG
 131  Q  A  L  A  L  M  Q  Q  Y  I  D  Y  K  K  E  L  V  L  L  E
1741 AACGCGACCTGCCGCGCCTGGCCGACCTCGACGCCCTGCGCCAGCGGGAAGCCGCGGTGA
 151  R  D  L  P  R  L  A  D  L  D  A  L  R  Q  R  E  A  A  V  K
1801 AAGCCCTGCGCGCGCGGATCTTCAGCAACGAAGCGCACGTGGCGTTCTTCGCCGACGAGG
 171  A  L  R  A  R  I  F  S  N  E  A  H  V  A  F  F  A  D  E  E
1861 AAACCTACAACCAGTTCACCCTGGAGCGCCTGGCGATCCGCCAGGATGGCAAGCTCAGCA
 191  T  Y  N  Q  F  T  L  E  R  L  A  I  R  Q  D  G  K  L  S  T
1921 CCGAGGAAAAGGCCGCCGCCATCGACCGCCTGCGCGCCAGCCTGCCGGAAGACCAGCAGG
 211  E  E  K  A  A  A  I  D  R  L  R  A  S  L  P  E  D  Q  Q  E
1981 AAAGCGTGCTGCCGCAACTGCAAAGCGAACTGCAGCAGCAGACCGCCGCCCTCCAGGCCG
 231  S  V  L  P  Q  L  Q  S  E  L  Q  Q  Q  T  A  A  L  Q  A  A
2041 CTGGCGCCGGCCCGGAAGCCATCCGCCAGATGCGTCAGCAACTGGTGGGCGCCGAAGCCA
 251  G  A  G  P  E  A  I  R  Q  M  R  Q  Q  L  V  G  A  E  A  T
2101 CCACCCGCCTGGAGCAACTCGATCGGCAACGCTCGGCCTGGAAGGGCCGGCTGGACGACT
 271  T  R  L  E  Q  L  D  R  Q  R  S  A  W  K  G  R  L  D  D  Y
2161 ATTTCGCCGAGAAGAGCCGGATCGAAGGCAATGCCGGGCTGAGCGAAGCCGACCGCCGCG
 291  F  A  E  K  S  R  I  E  G  N  A  G  L  S  E  A  D  R  R  A
2221 CGGCGGTCGAACGCCTGGCCGAGGAGCGCTTCAGCGAACAGGAACGCTTGCGCCTGGGCG
 311  A  V  E  R  L  A  E  E  R  F  S  E  Q  E  R  L  R  L  G  A
2281 CGCTGGAACAGATGCGCCAGGCCGAGCAGCGCTGACCGGCACGGAAACGCCGAGAACGCG
 331  L  E  Q  M  R  Q  A  E  Q  R
2341 GCGAAGGGCGCTTCGGCGGATAACGCTACCCTCAGGGGTGCAGCCCTGGCGTGGCCGGCG
2401 AGGCGGAAACCTGTGCTGCGCGCCGCAACGAAAAAGGGCGGCCACCCGAAGGTGTCCGCC
2461 CTTTTTCGTCGCCAGCCCGGTTCAGCGGGACAGCTTGCCGTCCAGCGAGAACTTGCCGGC
2521 GCCATCGATCAGCAGCGCCACGCTGATCATCAGCAGGGTCAGGGCATATTCATAGCCGTT
2581 GTCGGTGATGAAGAAGCCATTGCCGATGTGCACGCTGAAGATCGCCACGATCCGTTGACC
2641 TGCAGGTCGACCCAGATC
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Fig. 1-2. Nucleotide sequence of *lipA* and *lipB* genes from *Pseudomonas aeruginosa* TE3285. Putative Shine-Dalgarno sequences are boxed. Filled triangle indicates the position of the release of the signal peptide. Horizontal arrows indicate palindromic sequences, which are putative termination signals. The *lipB* sequence reported by Chihara-Siomi *et al.* (1992) has been corrected, and new sequence has been incorporated into the DDBJ/EMBL/databases with accession number of AB008452.

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                    1       10        20        30        40
TE3285 LipB         --MKKILLLIPLAFAASLAWFVWLEP----SPAPETAPPASPQAGADRAPPAASA
109 LimL            --MKKILLLIPLAFAASLAWFVWLEP----SPAPETAPPASAQAGADRAPPAAST
PAO1 LipH           ------------------------------------------------------
DSM3959 LimA        --MTARGGRAPLARRAVVYGAVGLAAIAGVAMWSGAGRHGGTGASGEPPDASAAR
KWI56 Act           --MTSREGRAPLARRAVVYGVVGLAAIAGVAMWSGAGWHRATGASGESPEASVAG
M-12-33 LipX        --MASRDG--------------------------------HGRRVAG-----RGSAG
PG1 LipB            MAQADRPARGGLAARPMRGASFALAGLVACAACAAVVLWLRPAAPSPAPAGAVAG

          50                60        70        80        90       100
LipB        GEAVPAP-------QVMPAKVAPLPTSFRGTSVDGSFSVDASGNLLITRDIRNLFDYFLSAVG
LimL        GEAVPAP-------QVMPAKVAPLPTSFRGTSVDGSFSVDASGNLLITRDIRNLFDYFLSAVG
LipH        ---------------MPAKVAPLPTSFRGTSVDGSFSVDASGNLLITRDIRNLFDYFLSAVG
LimA        GPAAAPP-------QAAVPASTSLPPSLAGSSAP-RLPLDAGGHLAKARAVRDFFDYCLTAQS
Act         GSVTAPP-------QAAVPASTGLPPSLAGSSAP-RLPLDAGGHLAKSRAVRDFFDYCLTAQS
LipX        GAAAAPP-------QAALPASTGLPSSLAGSSAP-RLPLDAGGHLAKSRAVRDFFDYCLTAQS
LipB(PG1)   GPAAGVPAAASGAAEAAMPLPAALPGALAGSHAP-RLPLAAGGRLARTRAVREFFDYCLTAQG
                                 **    *          * * *   *   *  *** * *

            110       120       130       140       150       160
LipB        EEPLQQSLDRLRAYIAAELQEP-ARGQALALMQQYIDYKKELVLLERDLPRL--A-DLDALRQ
LimL        EEPLQQSLDRLRAYIAAELQEP-ARGQALALMQQYIDYKKELVLLERDLPRL--A-DLDALRQ
LipH        EEPLQQSLDRLRAYIAAELQEP-ARGQALALMQQYIDYKKELVLLERDLPRL--A-DLDALRQ
LimA        DLSAAGLDAFVMREIAAQLDGTVAQAEALDVWHRYRAYLDALAKLRDAGAVD--KSDLGALQL
Act         DLSAAGLDAFVMREIAAQLDGTVAQAEALDVWHRYRAYLDALAKLRDAGAAD--KCDLGALQL
LipX        DLSAAALDAFVVRQIAAQLDGTVAQAEALDVWHRYRAYLDALAKLRDAGAVD--KSDLGALQL
LipB(PG1)   ELTPAALDALVRREIAAQLDGSPAQAEALGVWRRYRAYFDALAQLPGDGAVLGDKLDPAAMQL
                           *** *    *   **      *  *   *  *           *  *

               170       180       190       200       210       220
LipB        REAAVKALRARIFSNEAHVAFFADEETYNQFTLERLAIRQDGKLSTEEKAAAIDRLRASLPED
LimL        REAAVKALRARIFSNEAHVAFFADEETYNQFTLERLAIRQDGKLSAEEKAAAIDRLRASLPED
LipH        REAAVKALRARIFSNEAHVAFFADEETYNQFTLERLAIRQDGKLSAEEKAAAIDRLRASLPED
LimA        ALDQRASIAYRWLGDWS-QPFFGAEQWRQRYDLARLKIAQDPALTDAQKAERLAALEQQMPAD
Act         ALDQRASIAYRTLGDWS-QPFFGAEQWRQRYDLARLKIAQDPTLTDAQKAERLAALEQQMPAD
LipX        ALDQRASIAYRTLGDWS-QPFFGAEQWRQRYDLARLKIAQDRTLTDAQKAQRLAALEQQMPAD
LipB(PG1)   ALDQRAALADRTLGEWA-EPFFGDEQRRQRHDLERIRIANDTTLSPEQKAARLAALDAQLTPD
                       *         **  *       * * * *  *    **      *     *

            230       240       250       260       270       280
LipB        -QQESVLPQLQSELQQQTAALQAAGAGPEAIRQMRQQLVGAEATTRLEQLDRQRSAWKG----
LimL        -QQESVLPQLQSELQQQTAALQAAGAGPEAIRQMRQQLVGAEATTRLEQLDRQRSAWKG----
LipH        -QQESVLPQLQSELQQQTAALQAAGAGPEAIRQMRQQLVGAEATTRLEQLDRQRSAWKG----
LimA        ERAAQQRVDRQRAAIDQIAQLQKSGATPDAMRAQLTQTLGPEAAARVAQMQQDDASWQR----
Act         ERAAQQHIDQQRAAIDQIAQLQESGATPDAMRAQLTQTLGPEAAARVAQMQQDDASWQS----
LipX        ERAAQQRVDQQRAAIDRIAQLQKSGATPDAMRAQLTQTLGPEAAARVAQMQQDDASWQSATRT
LipB(PG1)   ERAQQAALHAQQDAVTKIADLQKAGATPDQMRAQIAQTLGPEAAARAAQMQQDDEAWQT----
                      *          * **  ** *    *     *  * **  *  *        *

            290       300       310       320       330       340
LipB        -----RLDDYFAEKSRIEGNAGLSEADRRAAVERLAEERFSEQER--LRLGALEQMRQAEQR
LimL        -----RLDDYFAEKSRIEGNTGLSEADRRAAVERLAEERFSEQER--LRLGALEQMRQAEQR
LipH        -----RLDDYFAEKSRIEGNTG-AERSRPPRGGRNAWPRSASANRNACAWARWEQMRQAEQR
LimA        -----RYADYAAQR-AQIESAGLSPQDRDAQI-AALRQRVFTKPGEAVR-AASLDRGAGSAR
Act         -----RYADYAAQR-TQIESAGLSPQDRDAQI-AALRQRVFTRPGEAVR-AASLDRGAGSAR
LipX        MRRSVRRSSRPACR-RRIATP-RSPHCGSARS-RNPAKRCGRHRSIAARGSAAVTRAARCA-
LipB(PG1)   -----RYQAYAAER-DRIAAQGLAPQDRDARI-AQLRQQTFTAPGEAIR-AASLDRGAGG--
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Fig. 1-3. Amino acid sequence alignment among *Pseudomonas* lipase activator proteins. Conserved residues are indicated with asterisk. The numbering of amino acids is given for *P. aeruginosa* TE3285 lipase activator protein (LipB). *P. aeruginosa* TE3285, (Chihara-Siomi *et al.*, 1992); *Pseudomonas* sp. 109, (Ihara *et al.*, 1992); *P. aeruginosa* PAO1, (Wohlfarth *et al.*, 1992); *P. cepacia* DSM3959, (Jørgensen *et al.*, 1991); *Pseudomonas* sp. KWI-56, (Iizumi *et al.*, 1991); *P. cepacia* M-12-33, (Nakanishi *et al.*, 1991); *P. glumae* PG1, (Frenken *et al.*, 1993).


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              1       10        20        30        40        50        60
TE3285      -STYTQTKYPIVLAHGMLGFDNILG-VDYWFGIPSALRRDGAQVYVTEVSQLDTS---EVRGEQL
109         -STYTQTKYPIVLAHGMLGFDNILG-VDYWFGIPSALRRDGAQVYVTEVSQLDTS---EVRGEQL
PAO1        -STYTQTKYPIVLAHGMLGFDNILG-VDYWFGIPSALRRDGAQVYVTEVSQLDTS- -EVRGEQL
DSM3959     AAGYAATRYPIILVHGLSGTDKYAGVLEYWYGIQEDLQQNGATVYVANLSGFQSDDGPNGRGEQL
KWI56       ADGYAATRYPIILVHGLSGTDKYAGVVEYWYGIQEDLQQNGATVYVANLSGFQSDDGANGRGEQL
M-12-33     ADNYAATRYPIILVHGLTGTDKYAGVLEYWYGIQEDLQQRGATVYVANLSGFQSDDGPNGRGEQL
PG1         ADTYAATRYPVILVHGLAGTDKFANVVDYWYGIQSDLQSHGAKVYVANLSGFQSDDGPNGRGEQL
                  *  **  * **  * *       ** **   *   ** ***   *         *****

                70        80        90       100       110       120
TE3285      LQQVEEIVALSGQPKVNLIGHSHGGPTIRYVAAVRPDLIASATSVGAPHKGSDTADFLRQIP--
109         LQQVEEIVALSGQPKVNLIGHSHGGPTIRYVAAVRPDLMPSATSVGAPHKGSDTADFLRQIP
PAO1        LQQVEEIVALSGQPKVNLIGHSHGGPTIRYVAAVRPDLIASATSVGAPHKGSDTADFLRQIP- -
DSM3959     LAYVKTVLAATGATKVNLVGHSQGGLSSRYVAAVAPDLVASVTTIGPADRGSEFADFVQDVLAYD
KWI56       LAYVKTVLAATGATKVNLVGHSQGGLTSRYVAAVAPDLVASVTTIGTPHRGSEFADFVQNVLAYD
M-12-33     LAYVKTVLAATGATKVNLVGHSQGGLTSRYVAAVAPDLVASVTTIGTPHRGSEFADFVQGVLAYD
PG1         LAYVKQVLAATGATKVNLIGHSQGGLTSRYVAAVAPQLVASVTTIGTPHRGSEFADFVQDVLKTD
            *  *    *  *  **** *** **   ****** * *  *  * *   **  ***

               130       140       150       160       170       180
TE3285      PGSAGEAILSGLVNSLGALISFLSSGSTGTQNSLGSLESLNSEGAARFNAKYP---HGVP TSAC
109         PGSAGEAVLSGLVNSLGALISFLSSGSAGTQNSLGSLESLNSEGAARFNAKYP---QGIP TSAC
PAO1        PGSAGEAVLSGLVNSLGALISFLSSGSTGTQNSLGSLESLNSEGAARFNAKYP---QGIP TSAC
DSM3959     PTGLSSSVIAAFVNVFGILTS--SSHNT-NQDALAALQTLTTARAATYNQNYPSAGLGAPGSCQT
KWI56       PTGLSSSVIAAFVNVFGILTS--SSHNT-NQDALAALQTLTTARAATYNQNYPSAGLGAPGSCQT
M-12-33     PTGLSSTVIAAFVNVFGILTS--SSNNT-NQDALAALKTLTTAQAATYNQNYPSAGLGAPGSCQT
PG1         PTGLSSTVIAAFVNVFGTLVS--SSHNT-DQDALAALRTLTTAQTATYNRNFPSAGLGAPGSCQT
            *           **  * * *  **      *  *  *  *      *  *  *   * *

               190       200                                 210       220
TE3285      GEGAYKVNGVS--YYSWSGS--SP---------------L-    -TNFLDPSD AFLGASSLTFK
109         GEGAYKVNGVS--YYSWSGS--SP---------------L - - TNFLDPSD AFLGASSLTFK
PAO1        GEGAYKVNGVS--YYSWSGS--SP---------------L---  TNFLDPSD AFLGASSLTFK
DSM3959     GAPTETVGGNTHLLYSWAGTAIQPTLSVFGVTGATDTSTLPLVDPANVLDLSTLALFGTGTVMIN
KWI56       GAPTETVGGNTHLLYSWAGTAIQPTLSVFGITGATDTSTVPLVDLANVLDPSTLALFGTGTVMIN
M-12-33     GAPTETVGGNTHLLYSWAGTAIQPTISVFGVTGATDTSTIPLVDPANALDPSTLALFGTGTVMVN
PG1         GAATETVGGSQHLLYSWGGTAIQPTSTVLGVTGATDTSTG-TLDVANVTDPSTLALLATGAVMIN
            *    * *     *** *   *                                 * * * *

               230       240       250       260       270       280
TE3285      NGT-ANDGLVGTCSSHLGMVIRDNYRMNHLDEVNQVFGLTSLFETSPVSVYRQHANRLKNASL
109         NGT-ANDGLVGTCSSHLGMVIRDNYRMNHLDEVNQVFGLTSLFETSPVSVYRQHANRLKNASL
PAO1        NGT-ANDGLVGTCSSHLGMVIRDNYRMNHLDEVNQVFGLTSLFETSPVSVYRQHANRLKNASL
DSM3959     RGSGQNDGLVSKCSALYGKVLSTSYKWNHLDEINQLLGVRGAYAEDPVAVIRTHANRLKLAGV
KWI56       RGSGQNDGLVSKCSALYGKVLSTSYKWNHLDEINQLLGVRGAYAEDPVAVIRTHANRLKLAGV
M-12-33     RGSGQNDGVVSKCSALYGQVLSTSYKWNHLDEINQLLGVRGANAEDPVAVIRTHANRLKLAGV
PG1         RASGQNDGLVSRCSSLFGQVISTSYHWNHLDEINQLLGVRGANAEDPVAVIRTHVNRLKLQGV
                  *** *  **   * *    *  ***** **  *         ** * * * ****
```

Fig. 1-4. Amino acid sequence alignment among lipases from *Pseudomonas* species. Conserved residues are indicated with asterisk. The numbering of amino acids is given for *P. aeruginosa* TE3285 lipase. *P. aeruginosa* TE3285, (Chihara-Siomi *et al.*, 1992); *Pseudomonas* sp. 109, (Ihara *et al.*, 1991); *P. aeruginosa* PAO1, (Wohlfarth *et al.*, 1992); *P. cepacia* DSM3959, (Jørgensen *et al.*, 1991); *Pseudomonas* sp. KWI-56, (Iizumi *et al.*, 1991); *P. cepacia* M-12-33, (Nakanishi *et al.*, 1991); *P. glumae* PG1, (Frenken *et al.*, 1992).

are believed to reactivate the lipase by means of a common mechanism. From analogy with other lipase activator proteins (Frenken *et al.*, 1993a, Jaeger *et al.*, 1994, Ihara *et al.*, 1995), LipB would be localized in the periplasm of the *Pseudomonas* cell, and anchored in the inner membrane by its N-terminal hydrophobic segment. Thus, in the cell, it is considered that unfolded LipA is permeated through the inner membrane in a signal peptide-dependent manner,

then this polypeptide is refolded by LipB in the periplasm following secretion into the extracellular medium.

With respect to genetic structure and *in vivo* function, LipB is likely to be similar to N-terminal propeptide of several proteases, such as subtilisin (Ikemura *et al.*, 1987), carboxypeptidase Y (Winther and Sørensen, 1991), and α-lytic protease (Silen *et al.*, 1989). These propeptides are synthesized as a single polypeptide with its mature protease region, and specifically mediate folding of the protease after translocation through cellular membrane (Shinde and Inouye, 1993, Eder and Fersht, 1995). From the crystallographic analysis of the complex between subtilisin and its propeptide (Gallagher *et al.*, 1995), the propeptide is likely to recognize specific structural elements of the mature region through the refolding process. Considering the functional similarity to the propeptides, also LipB could recognize some structural segments of LipA. Candidates of such substructures can be presumed by three-dimensional structures of bacterial lipases (Noble *et al.*, 1994, Lang *et al.*, 1996, Schrag *et al.*, 1997, Kim *et al.*, 1997). From these analyses, common structural features of bacterial lipases have been found, that is, (1) serine protease-like active site including catalytic triad and oxyanion hole, (2) hydrophobic surface on the walls of the active site cleft and helical lid structure for masking the hydrophobic site, and (3) calcium binding site. The first two features are also applicable to eukaryotic lipases according to their crystal structures, whereas the third one is fit to only bacterial lipases. Although three-dimensional structure of LipA from *P. aeruginosa* TE3285 has not been resolved, LipA is expected to possess the above three features. Thus, LipB could mediate construction of such structural features of LipA in its folding process. However, properties of LipB such as reactivation kinetics, requirements, and functional amino acid residues have been unknown because purification of LipB is difficult. It is considered that elucidation of its properties enables us to understand key process of protein folding in a molecular level. Furthermore, this could contribute to give an important insight into the protein translocation in the cell.

In the present study, the LipA reactivation mediated by LipB was analyzed in the molecular level by protein engineering techniques. In Chapter 2, LipB was overproduced in *E. coli*, and purified in the presence of detergent. By using this LipB sample, fundamental

characteristics of LipB were investigated by kinetic analysis of LipA reactivation and chemical cross-linking (Jaenicke and Rudolph, 1989) with denatured LipA. In Chapter 3, to investigate the characteristics of N-terminal region of LipB, two forms of N-terminal truncated LipBs were prepared, and their molecular properties and reactivation activity were compared with those of full-length form. In Chapter 4, to find important amino acid residues of LipB in reactivation, random mutagenesis using error-prone PCR (Leung *et al.*, 1989) was performed on a gene encoding the functional region of LipB. Given DNA library was introduced into lipase expression system using *E. coli*, and mutant LipB losing its activity was selected by two simple screening procedures. Consequently, five single-residue substituted LipBs were newly prepared, and their properties were compared with that of wild-type LipB.

CHAPTER 2

Overexpression, Purification, and Characterization of LipB, Lipase Activator Protein from *Pseudomonas aeruginosa* TE3285

2-1. Introduction

LipB, the lipase activator protein from *Pseudomonas aeruginosa* TE3285, is encoded at the position adjacent to the gene of its substrate LipA. Therefore, LipB would be a specific folding factor for LipA. In this respect, LipB seems to be more similar to N-terminal propeptide of several proteases such as subtilisin (Ikemura *et al.*, 1987) than to general molecular chaperones. In the case of subtilisin, calcium ion plays an important role in the propeptide-mediated folding. It has been considered that calcium binding to subtilisin during folding process is regulated by its propeptide (Gallagher *et al.*, 1995). This calcium binding contributes to the stability of the subtilisin structure, and so to the expression of its enzymatic activity. Bacterial lipases also have a calcium binding site that is important for maintaining their structure (Noble *et al.*, 1994, Lang *et al.*, 1996, Schrag *et al.*, 1997, Kim *et al.*, 1997). Thus, it could be presumed that LipB participates in this calcium binding during the reactivation process of LipA. However, the characteristics of LipB during reactivation have been little elucidated.

In this chapter, the recombinant full-length LipB was purified, and its function was kinetically analyzed *in vitro*. The purified LipB reactivated denatured LipA, but did not dissociate from the reactivated LipA *in vitro*. Furthermore, the effect of calcium ion on the LipB-assisted reactivation was examined, and it is proposed that LipB specifically assists the binding of calcium ion to LipA.

2-2. Experimental Procedures

Materials. Bacterial strain *Escherichia coli* BL21(DE3) [F^-, *ompT*, $hsdS_B(r_B^-m_B^-)$, *gal*, *dcm*, (DE3)] and plasmid pET-3d (Novagen Inc., Madison, WI) were used as a host and a vector (Studier *et al.*, 1990) for expression of *lipB*, respectively. Bacterial strain *E. coli* BW313 (*dut, ung*, *thi*-1, *relA*, *spo*T1/F'*lysA*) was used for the preparation of single strand DNA including uracil on site-directed mutagenesis (Kunkel, 1985). Plasmid pUL11 is a pUC19 derivative carrying both *lipA* and *lipB* from *Pseudomonas aeruginosa* TE3285 (Chihara-Siomi *et al.*, 1992). M13mp19 was provided by Takara Shuzo Co., Ltd. (Kyoto, Japan). Synthetic oligonucleotide was a gift from Toyobo Co., Ltd. (Osaka, Japan). Restriction enzymes and DNA modifying enzymes were obtained from Toyobo Co., Ltd. and Takara Shuzo Co., Ltd. The LipA protein purified from culture medium of *P. aeruginosa* TE3285 (Chihara-Siomi *et al.*, 1992) was a special gift from Toyobo Co., Ltd. The purified lipases from *Pseudomonas* sp. 109 (Ihara *et al.*, 1991) and *P. cepacia* M-12-33 were generous gifts from Nagase Biochemicals Ltd. (Tokyo, Japan) and Amano Pharmaceutical Co., Ltd. (Nagoya, Japan), respectively. The purified lipases from *Candida cylindracea* and porcine pancreas were obtained from Sigma Chemical Co (St. Louis, Missouri). Cellulofine GCL-1000m was a gift from Chisso Co. Ltd. (Tokyo, Japan). All other chemicals used in the present work were of the purest grade commercially available.

Plasmid construction. An expression plasmid of *lipB*, pELB10, was constructed as shown in Fig. 2-1. A *Kpn* I-*Bam*H I fragment of pUL11 containing *lipA* and *lipB* was inserted into M13mp19. By use of the given phage vector (M13D1) as a template, a new *Nco* I site was introduced at the position of the initiation codon of *lipB* by site-directed mutagenesis according to the method of Kunkel (1985). The mutational primer consists of the sequence 5'-GCCCCCTCCCATGGGGAAAATCCTCCTGC-3'. The plasmid pELB10 was prepared by isolating an *Nco* I-*Hin*d III fragment including *lipB* from the resulting phage vector (M13LB10), and inserting the fragment at the position downstream of the T7 promoter in pET-3d.

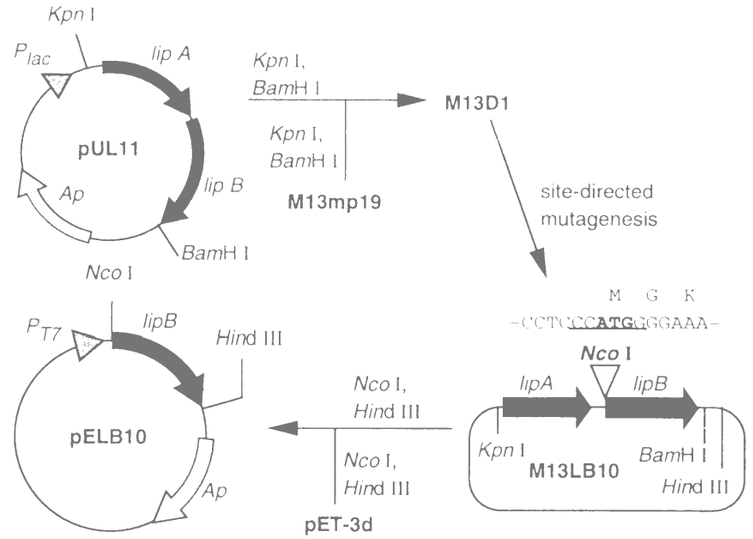

Fig. 2-1. Construction of pELB10, the expression plasmid of *lipB*.

Overproduction and purification of LipB. *E. coli* BL21(DE3) was transformed with the expression plasmid pELB10 and was cultured at 37 °C in 1 liter of Luria-Bertani broth (1% Bacto tryptone, 0.5% Bacto yeast extract, and 1% NaCl) containing 50 μg/ml ampicillin. Expression of *lipB* was induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside when the absorbance at 600 nm of the growing culture reached 0.7. After a total 5-h culture, bacterial cells were harvested by centrifugation at 7,000 × *g*, and cell pellets (3.5 g) were resuspended in 30 ml of the extraction buffer (20 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 5 mM $CaCl_2$, and 0.5 mM EDTA). The cell suspension was sonicated 20 times for 30 s at 0 °C using a Branson Sonifier 250, then centrifuged at 25,000 × *g* for 30 min at 4 °C, and the supernatant was recovered as crude extract. The fraction containing LipB was precipitated from the crude extract with 20 to 30% saturated ammonium sulfate. The precipitates were separated by centrifugation at 25,000 × *g* for 30 min at 4 °C and resuspended in the buffer (20 mM Tris-HCl, pH 8.0, 0.1 M NaCl, and 0.5 mM EDTA). The solution was dialyzed against the same buffer. The dialyzate was mixed with one-fourth volume of 10% SDS and applied to a Cellulofine GCL-1000m gel filtration column (2.6 × 90 cm) equilibrated with the eluent (20 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 0.5 mM EDTA, and 1% SDS) at room temperature. The ac-

tive fractions including 1.5 mg proteins were applied to SDS polyacrylamide gels (180 × 180 × 2 mm) and electrophoresed as described by Laemmli (Laemmli, 1970) at 20 °C. The gels were stained with Copper Stain (Bio-Rad Laboratories, Inc., Hercules, CA) (Lee *et al.*, 1987), and a band including LipB was excised from the gels. The purified protein was recovered by electroelution (Findlay, 1990). The eluate was applied to an Extracti-Gel D column (Pierce Chemical Co., Rockford, IL) to remove SDS.

Denaturation of LipA. Denatured LipA was prepared by addition of 48 μM native LipA solution (20 mM Tris-HCl, pH 8.0 and 0.2 M NaCl) to three volumes of the same buffer containing 8 M guanidine hydrochloride, followed by incubation for more than 1 h at room temperature. The denaturation of LipA was checked by examination of the circular dichroism in the range between 200 and 240 nm.

Assay of lipase activity. Protein concentration was determined with BCA Protein Assay Reagent (Pierce Chemical Co.) (Smith *et al.*, 1985). Bovine serum albumin was used as a standard. Lipase activity was measured with Lipase Kit S (Dainippon Pharmaceutical Co., Ltd., Osaka), in which 2,3-dimercaptopropan-1-ol tributyrate was used as a substrate (Kurooka *et al.*, 1977). One unit (U) was defined as an amount of lipase catalyzing the hydrolysis of one μmol of the ester to butyric acid per minute at 30 °C.

Assay of reactivation of the denatured LipA. The standard reactivation reaction was measured under the following conditions. The reactivation was initiated by diluting 5 μl of the denatured LipA solution (12 μM) into 200 μl of an appropriate concentration of the LipB solution including 20 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 5 mM $CaCl_2$, 0.5 mM EDTA, and 0.5% Triton X-100. After incubation at 20 °C for a given period, an aliquot of the mixture was withdrawn and the lipase activity was measured immediately with Lipase Kit S.

Cross-linking between LipA and LipB. Samples for chemical cross-linking were prepared as follows: 40 μl of 12 μM denatured LipA solution (20 mM triethanolamine-HCl, pH 8.0, 0.2 M NaCl, and 6 M guanidine hydrochloride) was mixed with 1.6 ml of LipB solution in 20 mM triethanolamine-HCl, pH 8.0, 0.2 M NaCl, 5 mM $CaCl_2$, 0.5 mM EDTA, and 0.5% Triton X-100, and was incubated for 4 h at 20 °C. The cross-linking reaction was carried out by addition of 5 μl of 70% glutaraldehyde to the sample mixture and incubation for 5 min at 20 °C (Jaenicke and Rudolph, 1989). The reaction was then quenched by adding 40 μl of 2 M sodium borohydride dissolved in 0.1 M NaOH. After the 20-min incubation, protein mixtures were precipitated with 7% trichloroacetic acid (Bensadoun and Weinstein, 1976), and analyzed by SDS-PAGE (Laemmli, 1970).

2-3. Results

Overexpression of lipB in E. coli. LipB was overproduced by using the T7 expression system (Studier *et al.*, 1990). An expression plasmid for *lipB*, pELB10, was constructed by inserting *lipB* at the position downstream of the T7 promoter in pET-3d (Fig. 2-1). The plasmid pELB10 was introduced into *E. coli* BL21(DE3), and the transformant was grown with induction by isopropyl β-D-thiogalactopyranoside. Crude extract of the cultured cells was analyzed by SDS-PAGE (Fig. 2-2, lane 2). A 37-kDa protein was detected only for the induced cells, and its molecular weight is consistent with that calculated from the DNA sequence of *lipB* (37.7 kDa). The 37-kDa protein fraction was blotted onto a polyvinylidene difluoride membrane, and its N-terminal amino acid sequence was analyzed by a gas-phase sequencer (Applied Biosystems Model 477A). This protein has the N-terminal sequence Met-Gly-Lys-Ile-Leu, as predicted from the DNA sequence of *lipB* (Fig. 1-2) except for the second Gly; this Gly is substituted for Lys of the native LipB owing to the site-directed mutagenesis. The expression level of LipB was estimated as 25% of the total cell proteins on the basis of densitometric analysis of the gels stained with Coomassie Brilliant Blue R-250.

Purification of LipB. LipB was purified 4.9-fold from the crude extract in three steps; ammonium sulfate fractionation, gel filtration chromatography, and polyacrylamide gel electrophoresis (Table 2-1). The SDS-PAGE analysis of each purification step is shown in Fig. 2-2.

Table 2-1. Purification of recombinant full-length LipB. Specific activity of LipB was determined from the initial rate of reactivation of denatured LipA. One unit of LipB was defined as the amount of LipB reactivating one unit of the denatured LipA per minute under the standard assay conditions.

Purification step	Total protein (mg)	Total activity (U)	Yield (%)	Specific activity (U/mg)	Purity (-fold)
Crude extract	310	827	100	2.64	1.0
Salting-out	80	483	58	6.05	2.3
Gel filtration	38	421	51	11.0	4.2
SDS-PAGE	5.8	75.1	9.1	12.9	4.9

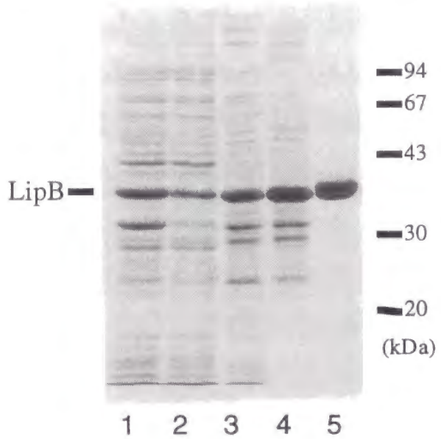

Fig. 2-2. SDS-PAGE analysis of the purification of LipB. A 12.5% polyacrylamide gel was used, and proteins were stained with Coomassie Brilliant Blue R-250. Lane 1, total cells; lane 2, crude extract; lane 3, ammonium sulfate fraction; lane 4, Cellulofine GCL-1000m eluate; lane 5, preparative SDS-PAGE fraction.

SDS was added to protein solutions and buffers at the steps of gel filtration and electrophoresis during the purification process. Without SDS, LipB was eluted in the void volume on gel filtration using Cellulofine GCL-1000m, of which the exclusion limit is 500 kDa, and it could not be separated from the other proteins. These results indicate that LipB readily forms large soluble aggregates. When LipB solution containing 1% SDS was diluted 500-fold into the reactivation buffer, its reactivation activity was the same as that of LipB without SDS treatment. Thus, it is likely that SDS effectively disperses the aggregates and does not inactivate LipB irreversibly.

Reactivation kinetics of LipB. Denatured LipA gradually recovered its lipase activity in the presence of LipB, whereas no lipase activity was recovered without LipB (Fig. 2-3). In

the presence of 0.28 μM LipB (at the LipB/LipA molar ratio of 1:1), the recovery of the lipase activity reached maximum at least 1 h after the initiation of the reactivation. When the concentration of LipB was lower than that of the denatured LipA, the maximum recovery increased with the amount of LipB. However, the maximum recovery of the lipase activity was inhibited by an excess amount of LipB. At LipB concentrations lower than 8.8 nM (at a LipB/LipA molar ratio of 1:30), no more than 14.7 U of the lipase activity was recovered by one nanomole of LipB. Since the activity of 1 nM native LipA is 34.3 U/l, LipB was expected to reactivate less than an equimolar amount of the denatured LipA. Thus, multiple turnover is not involved in the LipB-assisted reactivation of the denatured LipA. Moreover, reactivation was observed in the same manner when the purified LipA denatured with additional thiol reagents or the recombinant LipA produced as inclusion bodies in *E. coli* (Oshima-Hirayama *et al.*, 1993) was used for a substrate of LipB (data not shown). In addition, it was observed that the reactivated LipA possesses hydrolytic activity for triolein as well as 2,3-dimercaptopropan-1-ol used as the standard substrate in the present study.

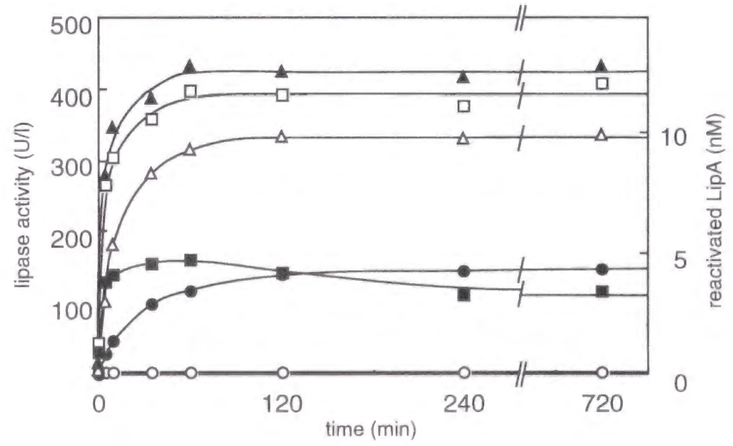

Fig. 2-3. Time course of the LipB-assisted reactivation of the denatured LipA. The denatured LipA solution was diluted 41-fold into LipB solution and incubated at 20 °C. The final concentration of the denatured LipA was 0.29 μM. The concentrations of LipB were as follows: 0.0 nM (○); 11 nM (●); 56 nM (△); 0.28 μM (▲); 1.4 μM (□); 7.1 μM (■).

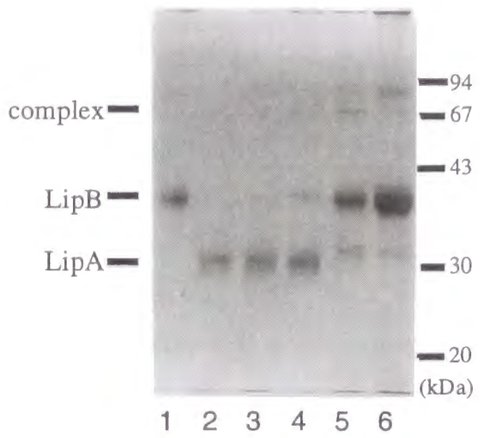

Fig. 2-4. SDS-PAGE analysis of the LipA-LipB complex cross-linked with glutaraldehyde. Protein mixtures on reactivation were treated with glutaraldehyde. A 12.5% polyacrylamide gel was used, and proteins were stained with Coomassie Brilliant Blue R-250. Lane 1, LipB; lane 2, denatured LipA; lanes 3-6, LipB and denatured LipA. The final LipA concentration was 0.29 μM. The concentrations of LipB were as follows: lane 3, 11 nM (at LipB/LipA molar ratio of 1:25); lane 4, 56 nM (1:5); lane 5, 280 nM (1:1); lane 6, 1400 nM (5:1). A band that does not permeate into the separating polyacrylamide gel is seen on lanes 1 and 3-6. These bands are considered to be cross-linked LipB aggregates. In addition, the 80-kDa band on lanes 1 and 6 is considered to be a dimerized LipB.

Cross-linking between LipB and reactivated LipA. It was thought that LipB would still be bound to LipA after the reactivation, since no multiple turnover was observed in the LipB-assisted reactivation (Fig. 2-3). To detect this LipA-LipB complex, chemical cross-linking experiments were carried out. A mixture of LipB and the denatured LipA was incubated for 4 h, then glutaraldehyde was added to the solution for cross-linking. The products were analyzed by SDS-PAGE (Fig. 2-4). A 68-kDa protein was newly observed (lanes 3-6), corresponding in size to the sum of LipA (30.1 kDa) and LipB (37.7 kDa). When either LipB or the denatured LipA was alone, no intermolecular cross-linked product was detected around the position of the 68 kDa protein (lane 1 or 2, respectively). These results indicate that the 68-kDa protein is the cross-linked 1:1 complex between LipB and LipA. This cross-linked product increased with the LipB concentration (lanes 3-5), but diminished with an excess amount of LipB (lane 6). These effects of the LipB concentration on the amount of the cross-linked product seem to be compatible with the effect of the LipB concentration on the recovery of lipase activity described above (Fig. 2-3). Thus, it is suggested that this complex detected by cross-linking expresses the lipase activity recovered with LipB.

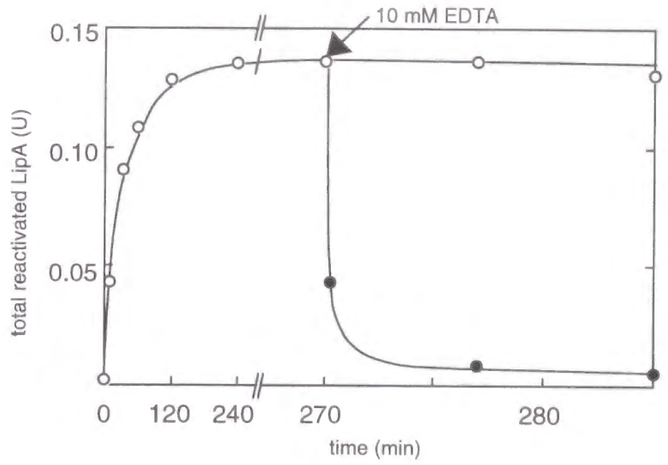

Fig. 2-5. Inactivation of the reactivated LipA by addition of EDTA. Reactivation was started by addition of 25 μl of the denatured LipA solution to 1 ml of 6 nM LipB solution at 20 °C under the standard condition. EDTA solution (final 10 mM) was added at 270 min from start of the reactivation. Total lipase activities are plotted for the reactivation mixture with EDTA (●) and without EDTA (○).

Effect of calcium ion. The effects of divalent cations on the LipB-assisted reactivation of the denatured LipA were examined. $CaCl_2$ was the most effective cation for the reactivation among those examined. The recovery of the lipase activity with 5 mM $MnCl_2$ was 59% of that with 5 mM $CaCl_2$, but in the presence of 5 mM $MgCl_2$, the reactivation was not observed. In the absence of divalent cations, the recovery of activity was undetectable. When 10 mM EDTA was added to the reaction mixture after 4.5-h reactivation, the recovered lipase activity was completely lost within 5 min (Fig. 2-5). In contrast, the native LipA was inactivated more slowly by the addition of EDTA; the activity of 3 nM LipA was reduced by half during 2-h incubation in the reactivation buffer with 10 mM EDTA (data not shown). Thus, LipA reactivated with LipB is more sensitive to inactivation by EDTA addition than the native LipA. Therefore, the effect of calcium ion on the complex formation between LipB and LipA was analyzed by cross-linking (Fig. 2-6). Without $CaCl_2$ the cross-linked band was not clearly detected (lane 2), indicating that calcium ion also affected the complex formation between

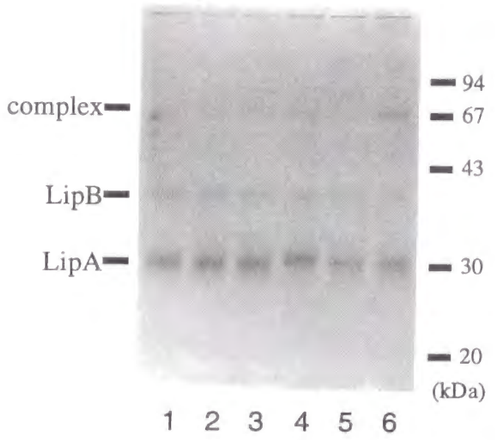

Fig. 2-6. SDS-PAGE analysis of the effect of calcium ion on the LipA-LipB complex. A 12.5% polyacrylamide gel was used, and proteins were stained with Coomassie Brilliant Blue R-250. The reactivation buffer contained Triton X-100 at the concentration of 0.5% (lane 1-3) or 0.05% (lane 4-6). For lanes 1 and 4, the 4-h reactivation reaction was carried out with 5 mM $CaCl_2$. For lanes 2 and 5, the reaction was carried out without $CaCl_2$. For lanes 3 and 6, the 4-h reactivation mixture with 5 mM $CaCl_2$ was further treated with 10 mM EDTA for 1 h. Protein mixtures were cross-linked with glutaraldehyde as described in Experimental Procedures.

LipB and LipA in the reactivation. Moreover, the cross-linked band was smeared (lane 3) when excess EDTA was added to the reactivation mixture that had been incubated sufficiently to allow the complex formation.

The absence of detergent in the reactivation assay lowered the recovery of the LipA activity to one-tenth of that with 0.5% Triton X-100. At least 0.05% Triton X-100 was needed to give the same level of reactivation as with 0.5% Triton X-100 (data not shown). At lower concentration of Triton X-100 (0.05%), without calcium ion, reactivation was not observed, and LipA-LipB complex formation was not clearly detected (Fig. 2-6, lane 5). The addition of EDTA also inactivated the lipase activity within 5 min in this case, though a considerable amount of the cross-linked product was observed (lane 6).

Substrate specificity of LipB. The substrate specificity of LipB in the reactivation was examined using denatured lipases from *Pseudomonas* sp. 109, *P. cepacia* M-12-33, *Candida cylindracea* and porcine pancreas. The final concentration of LipB was 14.2 nM, and that of the denatured lipase corresponded to 490 U/l of the native lipase. The denatured lipase from *Pseudomonas* sp. 109 was reactivated with LipB up to 205 U/l during 4-h incubation at 20 °C (without LipB: 2.0 U/l). In contrast, the denatured lipases from *P. cepacia* M-12-33, *Candida cylindracea* and porcine pancreas were little reactivated with LipB: the values of recovery (U/l) with LipB (without LipB) were 1.8 (5.7), 0.4 (0.0), and 0.7 (0.9), respectively.

2-4. Discussion

The full-length LipB of *P. aeruginosa* TE3285 has been purified, and the isolation of the purified LipB allowed the analysis of its molecular properties in the reactivation of LipA. The kinetic observations suggested that the reactivation reaction with LipB does not involve multiple turnover. The formation of the stoichiometric (1:1) complex between LipB and LipA was demonstrated by cross-linking experiments. By the addition of EDTA, this complex was readily dissociated, and its lipase activity was simultaneously lost. These phenomena suggested that LipA in the active form seems not to be released from LipB after reactivation *in vitro*, unlike molecular chaperones. Accordingly, some additional factors appear to be necessary for the release of the active LipA. To find such factors, the effects of several coenzymes on the LipB-assisted reactivation of the denatured LipA were examined. Neither the initial rate nor the maximum recovery in the reactivation was influenced by coenzymes such as ATP, GTP, NAD(P)H, and $NAD(P)^+$ (data not shown). However, the active LipA unbound with LipB is secreted into the extracellular medium of *Pseudomonas*. Consequently an unknown factor is anticipated to mediate the release of the active LipA from LipB during the secretion process in *Pseudomonas* cells. *Pseudomonas* has a characteristic complex of membrane proteins, Xcp proteins, which is considered to be an apparatus for the permeation of extracellular proteins through the outer cellular membrane (Tommassen *et al.*, 1992). Secretion of *Pseudomonas* lipase is also proposed to be mediated by Xcp proteins (Filloux *et al.*, 1987). Furthermore, it is observed that the lipase activator protein is located in periplasm (Frenken *et al.*, 1993a, Ihara *et al.*, 1995). Thus, one or more Xcp proteins are candidates for direct participation in the dissociation of the complex between LipB and the active LipA in periplasm.

It has been observed that the LipB-assisted reactivation is significantly stimulated by calcium ion. The complex formation of LipB with denatured LipA was found also to require calcium ion, since the cross-linked product between LipA and LipB was not detected in the absence of calcium ion. The LipA reactivated with LipB rapidly lost its enzymatic activity upon the addition of EDTA. These results suggest two possibilities for the function of calcium ion in the LipB-assisted reactivation of denatured LipA; one is that calcium ion is necessary for the interaction between LipA and LipB, and the other is that calcium ion is required

for the enzymatic activity of the reactivated LipA. Regarding the first possibility, calcium ion seems to participate in the formation of the LipA-LipB complex during the reactivation process. However, the removal of calcium ion by EDTA seems not necessarily to cause the dissociation of the complex, because the cross-linked product between LipA and LipB was not lost completely at a lower concentration (0.05%) of Triton X-100. Thus, calcium ion may indirectly play a role in the control of association and dissociation of the complex. On the other hand, the second possibility would be rather plausible, that is, calcium ion is needed to express the enzymatic activity of LipA refolded with LipB. It has been reported that the active conformation of *Pseudomonas* lipases is stabilized by calcium ion (Svendsen *et al.*, 1995). The calcium binding site of other bacterial lipases was determined by crystal structure analyses (Noble *et al.*, 1994, Lang *et al.*, 1996, Schrag *et al.*, 1997, Kim *et al.*, 1997). These lipases have two aspartic acid residues coordinated to the calcium ion. These residues are also conserved in LipA. Thus, LipA is believed to have a calcium binding site similar to that of the above bacterial lipases. In this respect, the reactivated LipA forming the complex with LipB is expected to differ from the native LipA in structure, because the reactivated complex was more rapidly inactivated by EDTA addition than the native LipA. Thus, it is suggested that LipB could affect this calcium binding to LipA in the reactivation process. This proposed function of LipB seems to be similar to that of the subtilisin propeptide as a protein-folding factor, since its refolding is also accompanied by calcium binding to the enzyme. The crystal structure of the propeptide-subtilisin complex suggests that the propeptide prevents the complete formation of the high-affinity calcium binding site of subtilisin until folding has occurred (Gallagher *et al.*, 1995).

It was found that LipB specifically recognizes and reactivates the denatured lipase from the same source. The lipase specificity of LipB appears to be related to the amino acid sequence of lipases since LipB also reactivates the *Pseudomonas* sp. 109 lipase, of which the amino acid sequence is 98% identical with that of LipA, but does not reactivate the denatured lipase from *P. cepacia* M-12-33 with 36% sequence identity to LipA. LipB reactivated neither of two eukaryotic lipases with little similarity to LipA in amino acid sequence. This strict specificity suggests that LipB recognizes a unique structural element of LipA during its folding process. In addition, regarding the sequence identity of the activator proteins, LimL from

Pseudomonas sp. 109 and LipX from *P. cepacia* M-12-33 are 98% and 37% identical with LipB, respectively. The extent of the identity among the activator proteins is similar to that among the corresponding lipases. Thus, the other lipase activator proteins could also essentially recognize and activate their own lipase. Hobson *et al.* (1993) suggested that lipase activator proteins function as a private chaperone for the *Pseudomonas* lipases based on their gene construction. The present results experimentally confirmed this idea of the specificity of the lipase activator protein.

LipB is considered to form soluble aggregates in buffer solution without detergents. By the addition of detergents to disperse the aggregates, the property of LipB was altered during the process of purification and during reactivation of the denatured LipA. These alterations seem to be attributed to an N-terminal hydrophobic segment of LipB, of which first 20 amino acid residues contain 17 hydrophobic residues. It is known that the LipB protein from *P. glumae* PG1 is anchored into the inner cellular membrane by its N-terminal hydrophobic segment (Frenken *et al.*, 1993a). In the case of the LimL protein from *Pseudomonas* sp. 109, its mutant protein lacking an N-terminal hydrophobic region can be separated by conventional chromatographic techniques without a detergent (Ihara *et al.*, 1995). On the basis of these observations, LipB from *P. aeruginosa* TE3285 is also expected to be buried in the cellular membrane at the hydrophobic N-terminal region. *In vitro*, LipB would be anchored by its N-terminal segment into detergent micelles, so that it would effectively disperse and reactivate the denatured LipA.

CHAPTER 3

Molecular Properties and Activity of Amino-Terminal Truncated Forms of LipB

3-1. Introduction

In the purification of full-length LipB, SDS was necessary for separating LipB from other proteins. Purified LipB did not reactivate even an equimolar amount of denatured LipA. From these observations it was suspected that full-length LipB readily forms soluble aggregates. It is considered that the N-terminal hydrophobic region of LipB cause aggregation, because in other lipase activator proteins, this N-terminal hydrophobic region is associated with the inner cellular membrane, and the main part of the molecule is in the periplasm, helping to refold the transported lipase (Frenken *et al.*, 1993a, Ihara *et al.*, 1995).

To identify characteristics of the N-terminal region, in this chapter, two forms of truncated LipB mutant proteins: Δ21LipB (LipB lacking the N-terminal 21 residues) and Δ61LipB (LipB lacking the N-terminal 61 residues) were prepared. Differences in the original and mutant proteins are shown in Fig. 3-1. It was observed that when N-terminal 21 residues were lacking, LipB was dispersed in solution and reactivated denatured LipA more effectively than full-length LipB. Furthermore, it was found that Δ61LipB reactivated denatured LipA.

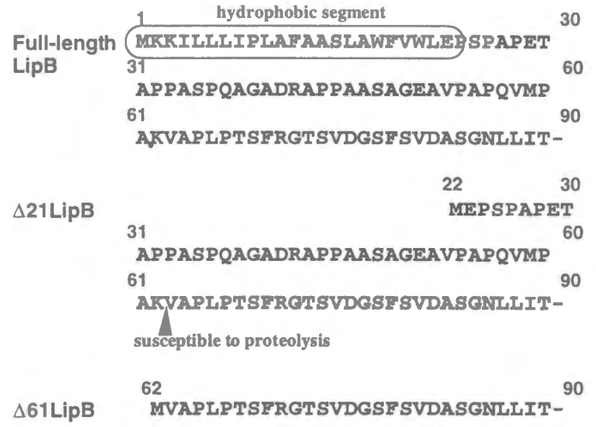

Fig. 3-1. N-terminal amino acid sequences of full-length and truncated LipBs.

3-2. Experimental Procedures

Materials. Bacterial strain *Escherichia coli* BL21(DE3) and plasmid pET-3d (Studier *et al.*, 1990) were used as the host and vector for production of the N-terminal truncated mutants of LipB, respectively. Synthetic oligonucleotides were obtained from Takara Shuzo Co., Ltd.

Plasmid construction. For expression of the gene of Δ61LipB, a plasmid named pELB31 was constructed. A new initiation codon with an *Nco* I site was introduced into the position encoding Lys62 of LipB by site-directed mutagenesis. The mutagenesis was done by the method of Eckstein (Taylor *et al.*, 1985) with the Sculptor *in vitro* mutagenesis system (Amersham, Buckinghamshire, UK). The mutational primer consists of the sequence 5'-GCCGGCCATGGTCGCGCC-3'. M13D1 was used as a template for the mutagenesis (Fig. 2-1). The gene of Δ61LipB was isolated from the resulting phage vector (M13LB30) digested by *Nco* I and *Hin*d III. The plasmid pELB31 was prepared by insertion of this fragment into pET-3d.

pELB20, an expression plasmid for the gene of Δ21LipB was designed in a similar way to pELB31. A new initiation codon was introduced into the position encoding Leu22 of LipB. The mutational primer consists of the sequence 5'-GGCCTGGTTCGTCTCCATGG-AACCTTCCCC-3'.

Production and purification of truncated LipBs. *E. coli* BL21(DE3) pELB31 was cultured for the production of Δ61LipB in the same way as for overproduction of full-length LipB in Chapter 2. From 6 liters of culture fluid, 20.4 g of cell pellets was obtained. These pellets were resuspended in 100 ml of the extraction buffer containing 20 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 0.5 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and were sonicated 20 times for 30 s at 0 ˚C with a Branson Sonifier 250. By centrifugation at 25,000 × *g* for 30 min at 4 ˚C, the supernatant was recovered as crude extract for later steps of purification.

Column chromatography was performed at 4 ˚C. The crude extract was mixed with an equal volume of the extraction buffer, which contains 2.0 M $(NH_4)_2SO_4$, and the mixture was centrifuged at 25,000 × *g* for 30 min at 4 ˚C. The supernatant was applied to a Butyl-

Toyopearl 650M column (2.6 × 30 cm) equilibrated with the starting buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1.0 M $(NH_4)_2SO_4$, and 0.5 mM PMSF). Proteins were eluted with a linear gradient from 1.0 to 0.0 M $(NH_4)_2SO_4$, and the active fraction was dialyzed against a mixture of 20 mM MES-NaOH, pH 6.5, 0.5 mM EDTA, and 0.5 mM PMSF. The dialyzate (12 ml) was mixed with 60 µl of 1 M $MnCl_2$, and applied to an AF-Red Toyopearl 650M column (1.6 × 7.5 cm) equilibrated with the starting buffer (20 mM MES-NaOH, pH 6.5, 0.5 mM EDTA, 5 mM $MnCl_2$, and 0.5 mM PMSF). After the column was washed with the same buffer for removal of unbound proteins, the active fraction was eluted stepwise with 20 mM MES-NaOH, pH 6.5, 10 mM EDTA, and 0.5 mM PMSF. The eluate was dialyzed against a mixture of 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, and 0.5 mM PMSF, and applied to a Q Sepharose FF column (2.6 × 20 cm) equilibrated with the same buffer. The active fraction was eluted with a linear gradient from 0.0 to 0.5 M NaCl. The purity of Δ61LipB in each step was checked by SDS-PAGE (Laemmli, 1970).

Δ21LipB was overproduced by the culture of *E. coli* BL21(DE3) pELB20, and purified from crude extract of the cells by the same protocol as Δ61LipB. However, for Δ21LipB, conditions for Butyl-Toyopearl column chromatography were slightly different: the crude extract was mixed with an extraction buffer that contains 1.6 M $(NH_4)_2SO_4$, and the Butyl-Toyopearl column was equilibrated with a buffer that contains 0.8 M $(NH_4)_2SO_4$.

The purified full-length LipB was prepared as described in Chapter 2.

Gel filtration analysis. HPLC gel filtration was done with a Tosoh instrument using a TSKgel G3000SW_{XL} PEEK column at 25 ˚C. The running buffer consists of 20 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 0.5 mM EDTA, and 0.02% NaN_3. The flow rate was 0.5 ml/min, and the elution of protein was detected by absorbance at 280 nm. The column was calibrated using Calibration proteins for gel chromatography (Boehringer Mannheim, Mannheim, Germany).

Assay of denatured LipA reactivation. The LipB-assisted reactivation of denatured LipA was carried out as described in Chapter 2.

3-3. Results

Overproduction and purification of truncated LipBs. Δ21LipB was overproduced with use of the T7 expression system in the same way as full-length LipB. Detergent was not required for the purification of Δ21LipB in any column chromatography, whereas SDS was necessary for the separation of full-length LipB from the other proteins.

When the purified Δ21LipB was stored without PMSF at 0 °C, Δ21LipB was readily digested, and a 30-kDa fragment was accumulated (data not shown). This fragment also reactivated denatured LipA. It was found that this fragment has an N-terminal sequence, Val-Ala-Pro-Leu-Pro, which is consistent with the sequence Val63-Pro67 of full-length LipB. Accordingly, a gene of LipB lacking the N-terminal 61 residues (Fig. 3-1) was prepared by site-directed mutagenesis. This mutant form, Δ61LipB, was overproduced and purified in the similar procedure as Δ21LipB. The SDS-PAGE after each purification step on Δ61LipB is shown in Fig. 3-2. The Δ61LipB was purified 35-fold with a yield of 20% (Table 3-1).

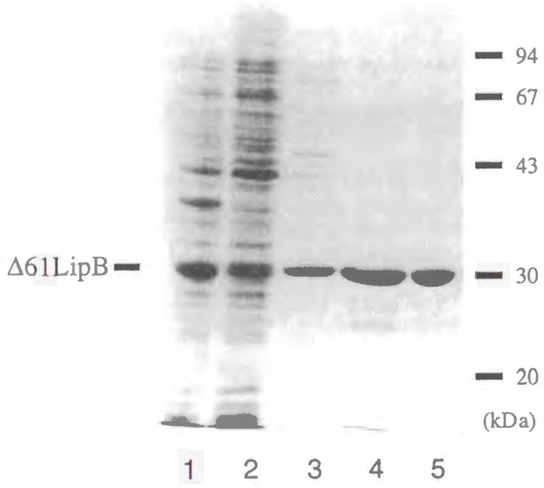

Fig. 3-2. SDS-PAGE analysis of the purification of Δ61LipB. A 12.5% polyacrylamide gel was used, and proteins were stained with Coomassie Brilliant Blue R-250. Lane 1, total cells; lane 2, crude extract; lane 3, Butyl-Toyopearl 650M eluate; lane 4, AF-Red Toyopearl 650M eluate; lane 5, Q Sepharose FF eluate.

Table 3-1. Purification of Δ61LipB. The specific activity of LipB was determined from the initial rate of reactivation of denatured LipA. One unit was defined as the amount of LipB reactivating one unit of denatured LipA per minute at 20 °C.

Purification step	Total protein (mg)	Total activity (U)	Yield (%)	Specific activity (U/mg)	Purity (-fold)
Crude extract	982	3660	100	3.73	1.0
Butyl-Toyopearl	38.9	2700	74	69.4	18.6
AF-Red Toyopearl	15.5	1480	40	95.5	25.6
Q Sepharose FF	5.57	727	20	131	35.1

Molecular dispersion of truncated LipBs. Because detergent was not needed in the purification of Δ21LipB or Δ61LipB, the molecular dispersion of these proteins in solution was examined by gel filtration analysis using TSKgel G3000SW$_{XL}$ PEEK column (Fig. 3-3). The exclusion limit of the column is 500 kDa. Full-length LipB was eluted in the void volume (6.5 ml) with a broad peak although monomers of this LipB have the molecular mass of 37.5 kDa. This finding indicates that full-length LipB forms large soluble aggregates. In contrast, Δ21LipB (35.3 kDa) was eluted in a sharp peak from gel filtration at the elution volume of 9.3 ml. This location corresponds to a molecular mass of 63 kDa, which is close to a dimeric size. Similarly, Δ61LipB (31.5 kDa) was sharply eluted at the elution volume of 9.9 ml, which corresponds to 50 kDa, close to a dimeric size. These results suggest that the truncation of at least the N-terminal 21 residues allows homogeneous dispersion of the molecules in solution.

Reactivation of denatured LipA with truncated LipBs. The reactivation of denatured LipA with full-length and two truncated LipBs was kinetically analyzed. Reactivation reached a maximum with 1 h of incubation with the truncated LipBs as with full-length LipB (Fig. 2-3). The maximum concentration of the reactivated LipA, which was determined after 4 h of incubation, is

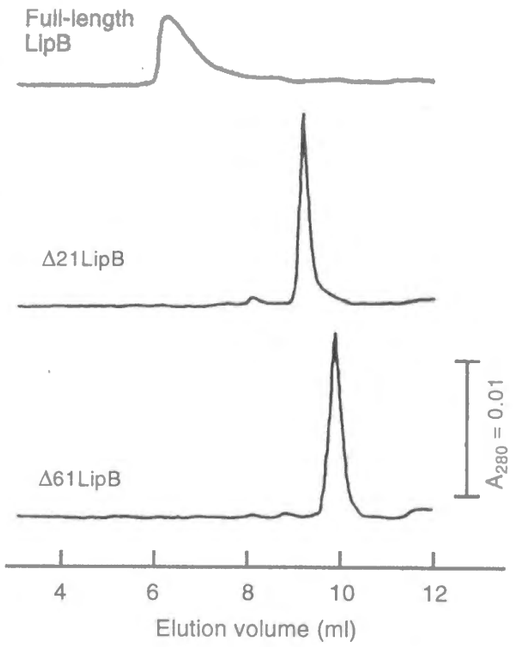

Fig. 3-3. Gel filtration of full-length and truncated LipBs. Ten microliters of purified LipB proteins (1 mg/ml) was applied to TSKgel G3000SW$_{XL}$ PEEK column. Elution of the proteins at the flow rate of 0.5 ml/min was monitored by the absorption at 280 nm.

plotted against the LipB concentration in Fig. 3-4. In all concentrations tested, either truncated LipBs recovered lipase activity much more effectively than the full-length LipB. When the concentration of the truncated LipB was less than 59 nM, 1 mole of LipA was fully reactivated per mole of LipB. In contrast, only 0.4 mole of LipA was reactivated per mole of full-length LipB even under the most suitable conditions with the LipB concentration less than 8.8 nM. These results indicate that the N-terminal 21 residues prevent LipB from the stoichiometric reactivation of the denatured LipA. This reactivation with truncated LipBs would involve no multiple turnover in the same way as full-length LipB.

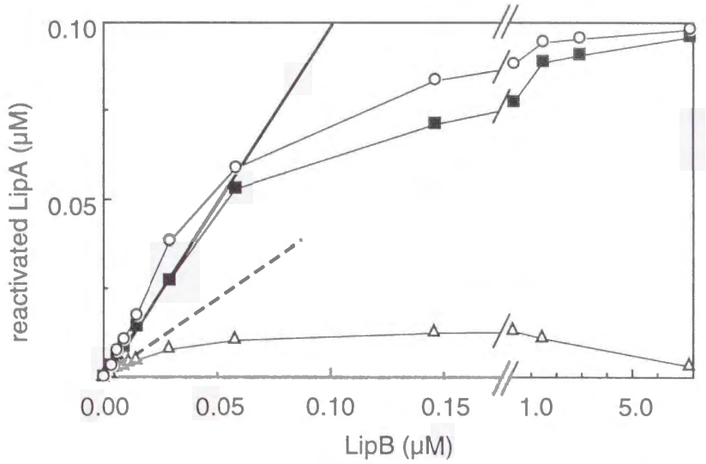

Fig. 3-4. Effects of the concentration of LipBs on maximum recovery of LipA activity. A solution of denatured LipA was diluted 41-fold in LipB solution (20 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 5 mM $CaCl_2$, 0.5 mM EDTA, and 0.5% Triton X-100) and incubated at 20 °C for 4 h. The final concentration of denatured LipA was 0.29 μM. The solid and dashed lines indicate theoretical results when 1.0 and 0.4 equimolar amounts, respectively, of denatured LipA are reactivated per mole of LipB. Full-length LipB (△); Δ21LipB (■); Δ61LipB (○).

3-4. Discussion

It was observed that truncation of the N-terminal region containing 21 residues affects the distribution of apparent molecular weight of LipB. The results of gel filtration suggest that the molecules forms soluble aggregates larger than 500 kDa, although the monomeric size is 37.5 kDa. The region containing the N-terminal 21 residues seems to cause aggregation of LipB molecules. In other lipase activator proteins, the N-terminal region of them is also hydrophobic (Jørgensen *et al.*, 1991, Ihara *et al.*, 1992, Frenken *et al.*, 1993a). The lipase activator protein of *P. glumae* PG1, is anchored in the inner membrane by its N-terminal hydrophobic segment, and acts on the lipase in the periplasm (Frenken *et al.*, 1993a). The N-terminal hydrophobic region of the lipase activator protein from *Pseudomonas* sp. 109, is considered to be a signal sequence for translocation into the periplasm (Ihara *et al.*, 1995). In this respect, the N-terminal region of LipB would be a similar membrane-associated segment. *In vivo*, because the N-terminal segment is anchored in the membrane or cleaved off, LipB could be effectively dispersed, and activate LipA in the periplasm.

The results of kinetic analysis show that truncation of the N-terminal 21 residues increases LipB-assisted reactivation of denatured LipA *in vitro*. Δ21LipB can quantitatively reactivate LipA although full-length LipB reactivated only 0.4 equivalent of LipA at the most. These results indicate that the N-terminal region of full-length LipB inhibits the reactivation activity of LipB *in vitro*. Thus, LipB would essentially reactivate a stoichiometric amount of denatured LipA. In addition, because the reactivation with Δ21LipB does not involve multiple turnover, Δ21LipB would complex with the reactivated LipA. This reactivated LipA is likely to have the same level of lipase activity as native LipA. In contrast, the lipase activator protein LimL from *Pseudomonas* sp. 109 was prepared as an N-terminal truncated mutant, and the complex between the lipase and the truncated LimL has a lower specific activity than the native lipase (Ihara *et al.*, 1995). Considering the behavior of LipB in the present study, LimL is expected to recover full activity of the lipase under certain conditions, such as in the presence of calcium ion.

It was further demonstrated that the N-terminal region of LipB containing 61 amino acid residues was not involved in its reactivation activity of denatured LipA. Δ61LipB is capable of reactivating the denatured LipA quantitatively, like Δ21LipB. Δ21LipB is readily digested between Lys62 and Val63, a position that may be exposed into the solvent. Thus, it is proposed that the region containing the N-terminal 62 residues of full-length LipB forms an independent domain from the residual C-terminal segment that reactivates LipA.

CHAPTER 4

Exploring Functional Amino Acid Residues of LipB by Random Mutagenesis

4-1. Introduction

Random mutagenesis is one of the useful methods for exploring functional amino acid residues of a protein. This method includes two processes: introduction of mutation into the gene of target protein, and subsequent screening of objective clones from the given mutational library. It is particularly important to establish a screening system suitable for the purpose to get successful results. Simple and effective screening procedures allow to identify the functional residues of target protein. Even after elucidation of three-dimensional structure of target protein, information deduced by the random mutagenesis must contribute to understanding its functional sites on the basis of the structure.

In Chapter 3, it was described that at least the N-terminal 61 residues of LipB was unnecessary for its reactivation activity. This finding indicates that Δ61LipB contains all of the functional amino acid residues required for the reactivation. For the purpose of exploring the functional residues, in this chapter, random mutagenesis was introduced into the gene coding for Δ61LipB by error-prone PCR (Leung *et al.*, 1989). Subsequently, inactive mutant LipBs losing reactivation activity were selected from the given library by two screening steps: plate assay with the guidance of active LipA secretion (Chihara-Siomi *et al.*, 1992), and detection of the molecular size of LipB by Western blotting to exclude nonsense mutants and frameshift ones. As a result, we found four amino acid residues that play an important role in the reactivation activity of LipB.

4-2. Experimental Procedures

Materials. Bacterial strain *Escherichia coli* 1100 (F^-, *prototrophic*, *endo* I^-) (Dürwald and Hoffmann-Berling, 1968) was used as the host for screening of inactive LipBs. *E. coli* JM109 (*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, λ^-, Δ(*lac-proAB*), [F', *proAB*, *lacI*^{q}*ZΔM15*, *traD36*]) and plasmid pRSET B (Invitrogen Co., San Diego, CA) were used for overproduction of mutant LipBs (Kroll *et al.*, 1993). pUL1 is a pUC19 derivative, in which *lipA* and *lipB* are cloned (Chihara-Siomi *et al.*, 1992). Synthetic oligonucleotides were obtained from Cruachem. (Kyoto, Japan). Restriction enzymes and DNA modifying enzymes were obtained from Toyobo Co., Ltd., Takara Shuzo Co., Ltd., and New England Biolabs, Inc. (Beverly, MA). Rabbit anti-Δ61LipB was purchased from Sawady Technology Co., Ltd. (Tokyo, Japan). Chelate Cellulofine was a gift from Chisso Co., Ltd.

Plasmid construction. A plasmid pULB200 was constructed for the template DNA on error-prone PCR for random mutagenesis (Fig. 4-1A). Plasmid pELB31 was digested by *Nco* I, treated with Klenow fragment, and digested by *Hin*d III. A 900-bp fragment containing Δ61LipB gene was ligated into pUC18 digested by *Sma* I*Hin*d III to give pULB200.

pUL32 was derived from pUL11 for expression of *lipA* in *E. coli* (Fig. 4-1B). First, a DNA fragment containing *lipB* with an *Nco* I site was isolated from M13LB30 digested by *Ppu*M I and *Hin*d III. The resulting fragment (1.1 kbp) was substituted for the corresponding segment of pUL11. The given plasmid, named pUL31, has two *Nco* I sites on *lipA* and *lipB*. Second, 430-bp of an *Xmn* I-*Spl* I fragment without *Nco* I site on *lipA* was isolated from pUL1, and ligated with fragments of pUL31 digested by *Xmn* I and *Spl* I. The resulting plasmid, pUL32, contains a single *Nco* I site on *lipB*, which encodes K62M mutant LipB.

pRSLB30 was constructed for overproduction of Δ61LipB as a fusion protein containing hexahistidyl leader sequence (Kroll *et al.*, 1993). The gene coding for Δ61LipB was isolated from pUL32 digested by *Nco* I and *Hin*d III, and inserted into pRSET B to obtain pRSLB30 (Fig. 4-1C).

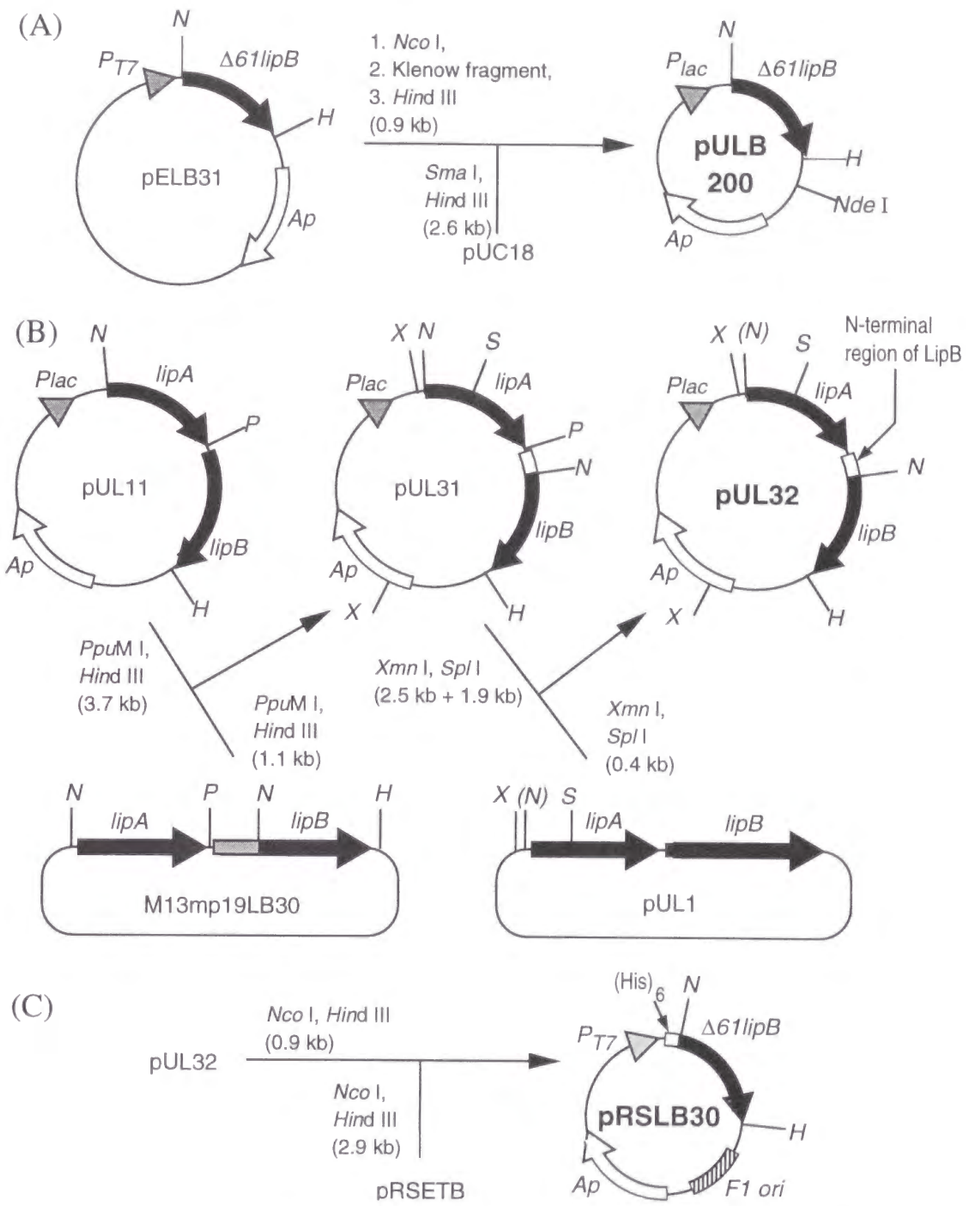

Fig. 4-1. Construction of the plasmids containing the gene coding for Δ61LipB. (A) pULB200 was used for the template of error-prone PCR on the gene coding for Δ61LipB. (B) pUL32 was the expression plasmid of *lipA*, and used for tributyrin plate assay, Western blotting for detection of LipB production, and DNA sequencing of the gene coding for Δ61LipB. (C) pRSLB30 was the expression vector for H6-Δ61LipB. Restriction sites are indicated as follows: *H*, *Hin*d III; *N*, *Nco* I; *P*, *Pml* I; *S*, *Spl* I; and *X*, *Xmn* I.

Random mutagenesis. Random mutation was introduced into the gene coding for Δ61LipB on the basis of error-prone PCR described by Leung *et al.* (1989) with some modifications. The linearized pULB200 (*Nde* I-digested) was used as the template DNA. Two synthetic oligonucleotides, 5'-TGTAAAACGACGGCCAGT-3' and 5'-CAGGAAACAGCTATGAC-3', were used as primers for amplification of the gene of Δ61LipB sequence. PCR was carried out using 10 ng of the template DNA, 40 pM each primer, 0.2 mM each dNTP, 1 × PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1.5 mM $MgCl_2$), 0.2-0.3 mM $MnCl_2$, 1% dimethyl sulfoxide, and 1.25 unit of *Taq* DNA polymerase in a total volume of 50 μl. PCR consists of 30 cycles using 30 s of denaturing at 94 ˚C, followed by annealing at 55 ˚C for 2 min and extension at 72 ˚C for 3 min. The PCR product was digested by *Nco* I and *Hin*d III, and a 900-bp fragment containing the gene coding for Δ61LipB was isolated. This fragment was ligated with the 3.9-kbp fragment of pUL32 digested by *Nco* I and *Hin*d III to produce a plasmid library.

Assay of LipB activity. *In vivo* activity of LipB produced in *E. coli* 1100 pUL32 was detected with the guidance of LipA secretion. Activity of secreted LipA was detected by hydrolysis of tributyrin on agar plate as described by Chihara-Siomi *et al.* (1992). In the present study, agar plates contain no isopropyl β-D-thiogalactopyranoside.

To detect *in vitro* activity of LipB, two kinds of assays; reactivation of denatured LipA and cross-linking with LipA by glutaraldehyde, were carried out as described in Chapter 2.

Western blotting. Cell harvest of 1 ml culture of *E. coli* 1100 pUL32 was solubilized and applied to SDS-PAGE according to Laemmli (1970). After the electrophoresis, separated proteins in the gels were blotted to polyvinylidene difluoride membrane. Then, the membrane was treated with Immun-Blot Assay Kit (Bio-Rad Laboratories). Rabbit anti-Δ61LipB and goat anti-rabbit IgG conjugated with horseradish peroxidase (Blake *et al.*, 1984) were used for the first and second antibody, respectively. Activity staining for peroxidase was performed with 4-chloro-1-naphthol and hydrogen peroxide as substrates.

DNA sequencing. Plasmids isolated from cell harvest of 3 ml culture of *E. coli* 1100 pUL32 were used for DNA sequencing on the gene coding for Δ61LipB. The sequencing was carried out by use of ABI PRISM Dye Terminator Cycle Sequencing Kit With AmpliTaq

DNA Polymerase, FS. DNA encoding C-terminal half and N-terminal half of Δ61LipB was sequenced with primer I (5'-TGTAAAACGACGGCCAGT-3') and II (5'-AGCAGCCTCCGCGGGAGAA-3'), respectively (Fig. 4-2). Electrophoresis and sequence analysis of the resulting samples were carried out by ABI PRISM 377 DNA sequencer.

Site-directed mutagenesis. Site-directed mutagenesis was carried out on the basis of the modification of the overlap extension PCR method (Ito *et al.*, 1991). Mutagenized pUL32 plasmid was used as the template. Primers described below are named according to Ito *et al.* (1991), and are schematically represented in Fig. 4-2. Primers I and II in the sequencing experiments were used for both rounds of PCR. Primer III (5'-CCTAGCTTGCATGCCTGCAGG-3') corresponds to the sequence located between primer I and the gene coding for Δ61LipB with one mismatched base that destroys the *Hin*d III site. Primers IV-a (5'-CGGAACCTGTTCGACTACTTCCTCAGC-3') or IV-b (5'-GAACCCCTTCAGCAAAGCCTG-3') were used for mutation in which the region encoding the amino acid residue of Arg94-Ser102 or Glu107-Leu113 is replaced by the wild-type sequence, respectively. These primers are designed to introduce a silent mutation with a restriction site: a *Bsp*E I site for primer IV-a, and an *Xmn* I site for primer IV-b. On the first PCR, two DNA fragments were amplified using either primers I and IV or primers II and III. After these products were mixed and annealed, the second PCR was performed with primers I and II. The resulting product was digested with *Nco* I and *Hin*d III, and then was substituted for the corresponding segment of pUL32.

Construction of plasmids encoding single-residue substituted Δ61LipB. From multiple-residue mutagenized plasmids, five single-residue substituted mutants (D76G, Y99C, Y99H, S102R, and R115C) were prepared as follows. The mutational sites of each mutant and restriction sites are illustrated in Fig.4-2.

(1) D76G. pUL32 (D76G/F100Y/F195L/I256T/R261H/D277G/R309C) was digested by *Nco* I and *Pml* I, and a 360-bp fragment containing the region of D76G/F100Y was inserted into the corresponding position of wild-type pUL32. Next, Tyr100 in the resulting plasmid was returned to phenylalanine residue by site-directed mutagenesis using the primer IV-a, so that pUL32 (D76G) was constructed.

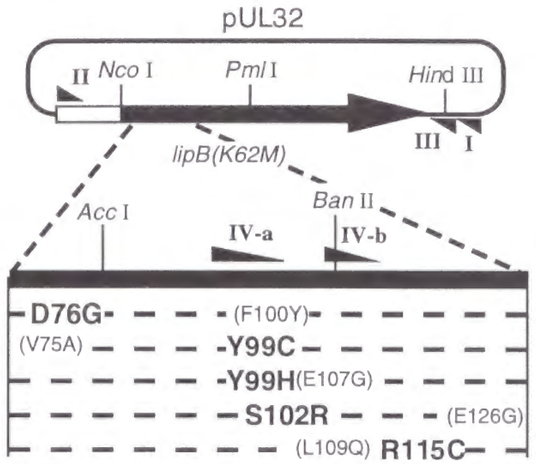

Fig. 4-2. Schematic drawing of restriction sites and primer locations in pUL32. Filled triangles show annealing regions of primers used in DNA sequencing and site-directed mutageneses. Five single-residue substituted LipBs are represented in bold letters. Residues replaced by that of the wild-type to prepare the single-substituted mutants are shown in parentheses with arranging in the respective line.

(2) Y99C. pUL32 (V75A/Y99C) was digested by *Acc* I, and an 820-bp fragment containing the region of Cys99 was inserted into the corresponding region of pULB200 with wild-type sequence of Δ61LipB. Then, a 900-bp *Nco* I-*Hin*d III fragment from the resulting plasmid was substituted for the corresponding segment of wild-type pUL32 to give pUL32 (Y99C).

(3) Y99H. pUL32 (Y99H) was prepared by site-directed mutagenesis of pUL32 (Y99H/E107G) using the primer IV-b. Gly107 was then replaced by glutamic acid residue.

(4) S102R. pUL32 (S102R/E126G) was digested by *Nco* I and *Hin*d III, and a 900-bp fragment was once inserted into the corresponding position of pULB200. This plasmid was digested by *Ban* II, and a 150-bp fragment with the region of Arg102 was substituted for the corresponding segment of wild-type pULB200. The resulting plasmid was then digested by *Nco* I and *Hin*d III, and a 900-bp fragment was replaced by the corresponding region of wild-type pUL32 to obtain pUL32 (S102R).

(5) R115C. pUL32 (R115C) was prepared by site-directed mutagenesis of pUL32 (L109Q/R115C) using the primer IV-b. Gln109 was then replaced by leucine residue.

Overproduction and purification of H6-Δ61LipB. For preparation of H6-Δ61LipB (Δ61LipB fusion protein containing hexahistidyl leader sequence), *E. coli* JM109 pRSLB30 was cultured in 100 ml of SOB medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 0.05% NaCl, 2.5 mM KCl, and 10 mM $MgCl_2$) containing 50 μg/ml ampicillin at 37 °C. When absorbance at 600 nm reached 0.3, 1 mM of isopropyl β-D-thiogalactopyranoside was added. After further 1-h culture, M13 phage including the gene of T7 RNA polymerase was mixed with the culture, and the cells were grown for 5 h.

Pellets of the cells were suspended in 10 ml of the extraction buffer containing 20 mM sodium phosphate buffer, pH 7.5, 0.2 M NaCl, 10 mM imidazole, 0.02% NaN_3, and 0.5 mM PMSF, and sonicated 10 times for 30 s at 0 °C with a Branson Sonifier 250. After centrifugation at 20,000 × *g* for 20 min at 4 °C, the supernatant was applied to a Ni^{2+}-bound Chelate Cellulofine affinity chromatography column (1.1 × 5 cm) equilibrated with the starting buffer (20 mM sodium phosphate buffer, pH 7.5, 0.5 M NaCl, 30 mM imidazole, 0.5 mM PMSF, and 0.02% NaN_3). The active fraction was eluted with 300 mM imidazole.

4-3. Results

Random mutagenesis and screening of inactive LipBs. To explore amino acid residues of LipB required for reactivation of LipA, random mutagenesis was carried out on the gene coding for Δ61LipB, and inactive LipB mutants were selected by two steps of screening, as summarized in Fig. 4-3. The mutation was introduced by error-prone PCR method (Leung *et al.*, 1989) using pULB200 as the template. Addition of 0.2-0.3 mM $MnCl_2$ to the PCR reaction mixture stimulated incorporation of incorrect nucleotides during the polymerization. The PCR products were inserted at immediate downstream of the N-terminal 61-residue region of LipB into pUL32 to prepare plasmid library (Fig. 4-1).

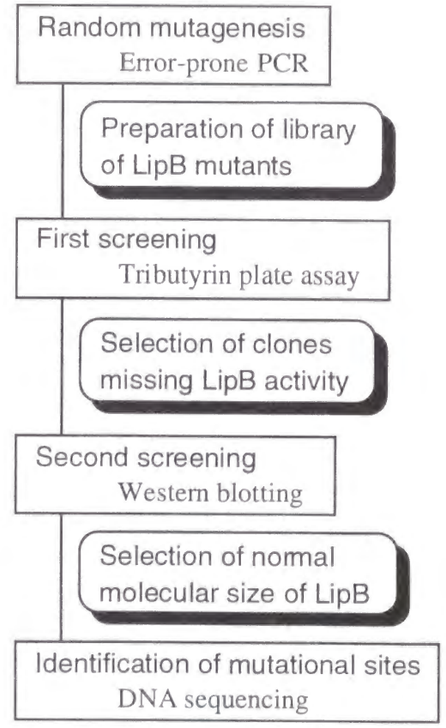

Fig. 4-3. Scheme of random mutagenesis and subsequent screening steps for obtain inactive forms of LipB.

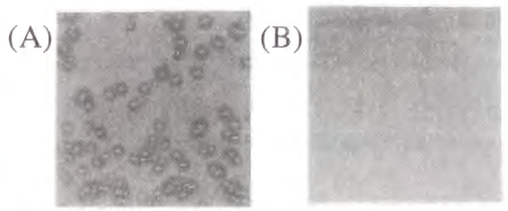

Fig. 4-4. Tributyrin plate assay for determining LipB activity in *E. coli* 1100 pUL32. (A) Clear zone-positive colonies that possess wild-type *lipB* sequence in pUL32. (B) Clear zone-negative colonies. These clones are expected not to produce active LipB.

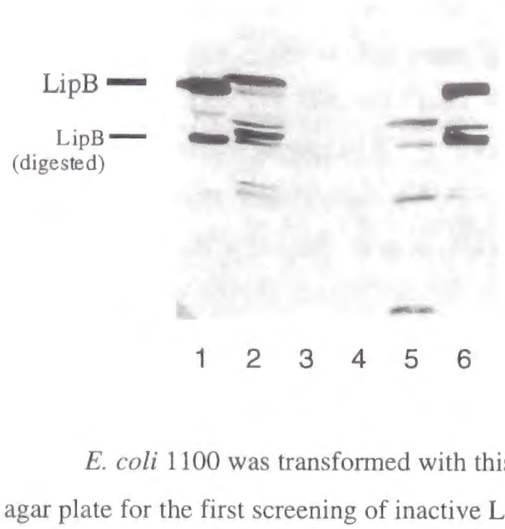

Fig. 4-5. Western blotting analysis of LipB production in *E. coli* 1100 pUL32. Lane 1, wild-type LipB; lanes 2-6, LipB from clear zone-negative clones. Sample on lane 6 was selected as a clone with producing a normal size LipB. Digested product was always detected even in the wild-type (lane 1).

E. coli 1100 was transformed with this pUL32 library, and was cultured on tributyrin agar plate for the first screening of inactive LipBs (Fig. 4-4). Colony of *E. coli* 1100 pUL32 with wild-type LipB formed clear zone due to hydrolysis of tributyrin by secreted active LipA. In the case of mutational library of pUL32, up to 20 colonies formed no clear zone out of about 3000 colonies from one plate (15 cm of diameter). Then, 175 of colonies without clear zone were picked up and cultured for the second screening to exclude frameshift mutants and nonsense ones. This screening was carried out by Western blotting with anti-Δ61LipB to detect the molecular weight of produced LipB in each cell. Some examples of this Western blotting analysis are shown in Fig. 4-5. On lane 6, a band was detected at the same position as wild-type LipB on lane 1. As a result, 27 types of such samples that contain the normal size of LipB were selected. Plasmids were isolated from these selected mutant cells, and were sequenced on the gene coding for Δ61LipB. In all of these mutants, multiple amino acids (2-16 residues) were replaced (Table 4-1).

Table 4-1. List of Δ61LipB mutants obtained by random mutagenesis.

No.	Amino acids	(Bases)	Mutational sites
30B	2	(2)	V75A Y99C
29A	2	(2)	Y99H E107G
30C	2	(3)	S102R E126G
30E	2	(2)	L109Q R115C
26H	3	(3)	S69P L146Q D289V
26E	3	(6)	F80S L287P E293G
26K	3	(5)	D98G F186L E317V
19G	3	(7)	L101F Y119N R314C
29B	3	(4)	E107G E145K E324G
27D	3	(4)	F186L F320C L328P
26M	3	(5)	T270S L315P L331P
30F	4	(4)	F97S A128T L220P E239G
26I	4	(5)	V104D R163H R327S E338V
26L	4	(7)	E107V L146R K294V N300Y
26D	5	(5)	V63A G77S T210I I257T E317V
26G	5	(7)	L101P S102G I140T K207G E305G
26C	6	(9)	P67L S69P S74C L96Q A216T S321G
26N	6	(9)	G72S S78C D92G L113Q K207E A245V
26B	6	(6)	S80G D92Y A171V F195S E230G R308S
26J	6	(7)	R91C P127L Q131R L162R L208P R314H
11B	6	(6)	Y99H S112G L153P Q229R L247P L331P
27C	6	(8)	Y139F S178R E211D A246T E274K L287P
27E	6	(7)	R165L A181V K207R Q263R L287P I297V
26A	7	(7)	D76G F100Y F195L I257T R261H D277G R309C
21A	10	(14)	I93V F100S S102R Q111L E180G L224P Q228R F291S E317G R340C
17C	12	(14)	S78R N86S E123K K170R L200P S209G L247P A250V M260V V265A E324G L331P
21C	16	(18)	L88P F97S E106V L109Q L133S L135P Y139C D160G N179D V183A K207V S209G S223G L240P A245T Q275R

Preparation of single-residue substituted inactive LipBs. Preparation of single-residue substituted LipBs from above mutants was attempted by genetic engineering techniques to determine functional residue for activity of LipB. A DNA fragment containing a part of the mutational sites was substituted for the corresponding segment of wild-type pUL32 to obtain a new plasmid with the other part of the mutational sites. In other cases, site-directed mutagenesis was performed to replace one of the mutational sites by wild-type sequence. New plasmids prepared in these procedures were introduced into *E. coli* 1100, and the clones of inactive LipB were further selected by tributyrin plate assay. The constructed plasmid was checked again by DNA sequencing. As a result, five genes of single-residue substituted mutant LipBs missing their *in vivo* activity (D76G, Y99C, Y99H, S102R, and R115C) were obtained from multiple-residue mutagenized plasmids, pUL32 (D76G/F100Y/F195L/I256T/R261H/D277G/R309C), pUL32 (V75A/Y99C), pUL32 (Y99H/E107G), pUL32 (S102R/E126G), and pUL32 (L109Q/R115C), respectively. The mutational positions of single-residue or double-residue substituted LipB finally obtained are illustrated in Fig. 4-6.

Preparation of the purified H6-Δ61LipB mutants. Purified samples of the five single-residue substituted mutants were prepared as hexahistidyl fusion proteins (H6-Δ61LipB) for examination of their *in vitro* properties. A DNA fragment encoding Δ61LipB

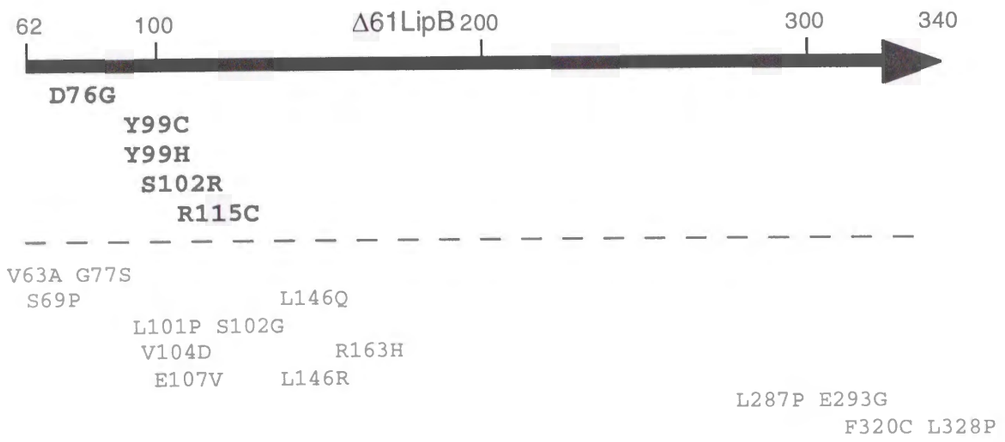

Fig. 4-6. Mutational sites of single- or double-residue substituted mutants of Δ61LipB. Substituted residues are identified by DNA sequencing of the gene coding for Δ61LipB.

mutant from pUL32 was inserted into an expression plasmid pRSET B. The resulting plasmid was introduced into *E. coli* JM109, and H6-Δ61LipB was overproduced. Five mutants of H6-Δ61LipB as well as the wild-type were readily purified with the Ni^{2+}-bound Chelate Cellulofine. Five mutant H6-Δ61LipBs were observed to show similar circular dichroism spectra to wild-type H6-Δ61LipB. Typical examples are shown in Fig. 4-7. Thus, the folding of each mutant LipB would not be significantly changed by substitution of the amino acid residue.

In the case of Y99C and R115C, effect of the substitution of cysteine residue on molecular assembly was analyzed by SDS-PAGE with or without 2-mercaptoethanol (Fig. 4-8). Without the reductant, both mutants appeared as two bands, which are close to the sizes of monomer (36 kDa) and dimer (72 kDa). In contrast, dimeric size of the band disappeared by addition of 2-mercaptoethanol. Because these mutants have only one cysteine residue introduced by the mutagenesis, parts of molecules of Y99C and R115C are likely to form dimers by the disulfide bond with the substituted cysteine residue, Cys99 and Cys 115, respectively.

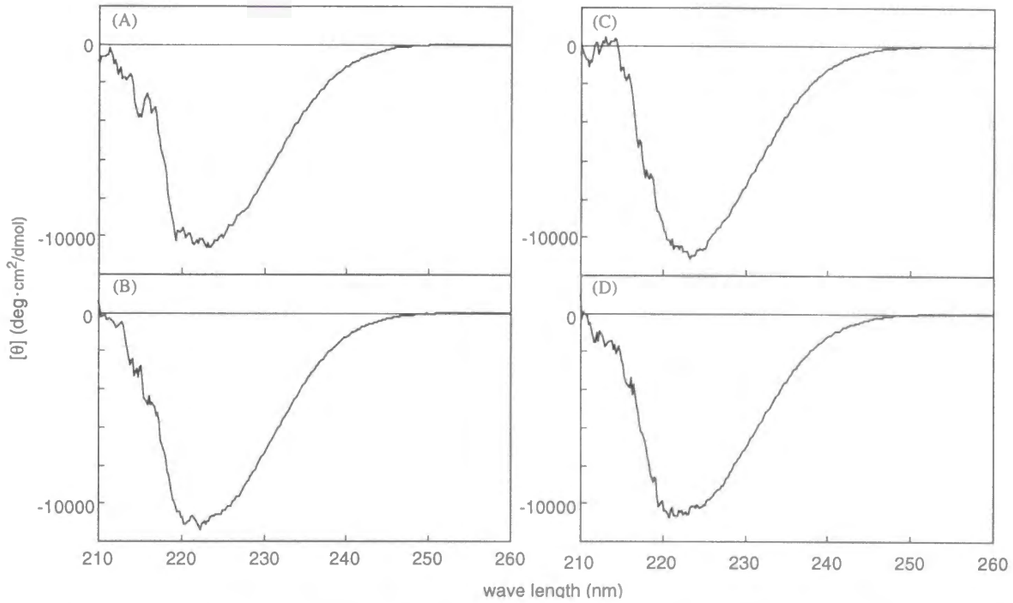

Fig. 4-7. Circular dichroism spectra of the wild-type and mutant H6-Δ61LipBs. (A) Wild-type; (B) Y99C; (C) S102R; (D) R115C.

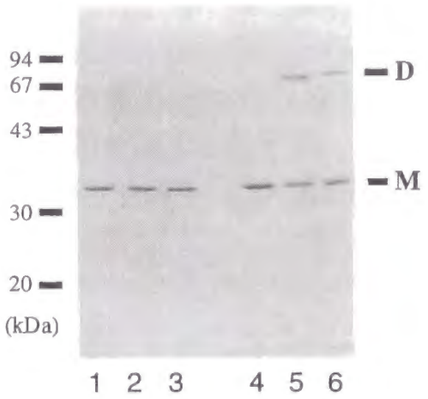

Fig. 4-8. SDS-PAGE analysis of the effect of 2-mercaptoethanol on molecular sizes of Y99C and R115C of H6-Δ61LipB. Lanes 1 and 4, the wild-type; lanes 2 and 5, Y99C; and lanes 3 and 6, R115C. The samples on lanes 1-3 were treated with 2-mercaptoethanol, whereas those on lanes 4-6 were not. The letters M and D represent monomeric and dimeric sizes of H6-Δ61LipB, respectively. A 12.5% polyacrylamide gel was used, and proteins were stained Coomassie Brilliant Blue R-250.

In vitro reactivation of mutant LipBs. Activity of single-residue substituted H6-Δ61LipB was determined by measurement of reactivation of denatured LipA. H6-Δ61LipB with the wild-type sequence was observed to recover lipase activity of denatured LipA in the same way as Δ61LipB. Activity of the mutant LipBs relative to the wild-type is shown in Table 4-2. The recovery of lipase activity with all the mutants was lower than that with wild-type H6-Δ61LipB: this result was consistent with the features in tributyrin plate assay. Notably, Y99C, Y99H, S102R, and R115C expressed less than 1% activity of wild-type H6-Δ61LipB in 4-h reactivation of denatured LipA. Thus, amino acid residues of Tyr99, Ser102, and Arg115 play a significant role in reactivation activity of LipB. In contrast, D76G recovered lipase activity that is about one third of the recovery with wild type H6-Δ61LipB. Initial rate of reactivation with D76G was observed to be 3% of that with wild-type H6-Δ61LipB.

Ability of the mutants to form a complex with denatured LipA was further examined by cross-linking with glutaraldehyde (Fig. 4-9). Wild-type H6-Δ61LipB was cross-linked with LipA after reactivation, and a 66-kDa band was detected by the SDS-PAGE. In contrast, Y99C, Y99H, S102R, and R115C, which almost lost reactivation activity, showed no detectable cross-linked product with LipA. Thus, these mutants altered to lose the ability to complex with LipA. In addition, D76G was slightly cross-linked with LipA.

Table 4-2. Reactivation of denatured lipase with H6-Δ61LipB mutants. Reactivation was started by addition of 5 µl of 12 µM LipA denatured with 6 M guanidine hydrochloride into 200 µl of LipB solution (20 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 5 mM $CaCl_2$, 0.5 mM EDTA, and 0.5% Triton X-100). The mixture was incubated at 20 °C, then lipase activity was measured. The relative lipase activities to the wild-type are shown.

Mutants	Initial rate [a] (%)	Recovery with 4-h reactivation [b] (%)
D76G	2.8	35.1
Y99C	N.D. [c]	0.21
Y99H	N.D.	0.08
S102R	N.D.	0.53
R115C	N.D.	0.51
wild-type	100	100

[a] Initial rate of reactivation during 5-min reactivation.

[b] Recovery of lipase activity by 4-h incubation. In the case of wild-type H6-Δ61LipB, reactivation reached maximum at the period.

[c] Not determined.

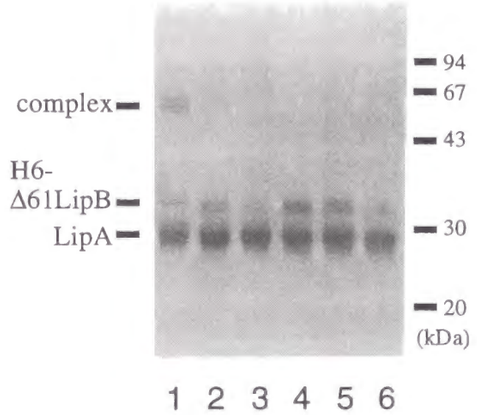

Fig. 4-9. Cross-linking between mutant H6-Δ61LipB and LipA with glutaraldehyde. After 4-h reactivation, glutaraldehyde was added to a mixture of H6-Δ61LipB (56 nM) and denatured LipA (290 nM). Lane 1, wild-type; lane 2, D76G; lane 3, Y99C; lane 4, Y99H; lane 5, S102R; and lane 6, R115C. A 12.5% polyacrylamide gel was used, and proteins were stained Coomassie Brilliant Blue R-250.

4-4. Discussion

To find the functional amino acid residues of LipB, random mutagenesis was carried out on the gene of Δ61LipB, and mutant LipBs missing the activity were selected, of which an amino acid residue is substituted. The random mutation itself is readily introduced by error-prone PCR (Leung *et al.*, 1989). Subsequently, two steps of screening methods were adopted to select the inactive LipBs from the given library: tributyrin plate assay in the first step and Western blotting to detect the molecular size of LipB in the second step. On the tributyrin plate, the secretion of lipase from bacterial colonies can be detected (Lawrence *et al.*, 1967). It has been observed that the active LipA was secreted by an *E. coli* strain carrying pUL11, a plasmid containing *lipB* as well as *lipA*, so that the colonies formed clear zone due to the (Chihara-Siomi *et al.*, 1992) hydrolysis of tributyrin . Furthermore, when the *lipB* gene was disrupted in the plasmid, the transformant did not secrete the active LipA, then the clear zone was not detected. By utilizing these features, the clones with inactive LipB were selected on tributyrin plate assay from the library of pUL32, a derivative of pUL11. However, a series of the clear zone-negative clones included the frameshift mutants and nonsense mutants (data not shown). It is reported that the error-prone PCR using $MnCl_2$ induces not only the base substitution but also the base deletion (Leung *et al.*, 1989). The frameshift or nonsense mutants do not fit the present purpose , that is, determining the amino acid residues important to the activity of LipB. It is expected that such clones would produce a different molecular size of LipB from the wild-type one. Thus, the LipB production in each cell selected in the first screening was subsequently analyzed by Western blotting using anti-Δ61LipB to exclude those unnecessary mutants. The selected mutants with the normal molecular size were observed to be base-substituted proteins by DNA sequencing experiments. Consequently, these simple screening steps have allowed rapid discrimination of functional amino acid residues of LipB.

In Chapter 3, it is demonstrated that the N-terminal 61 residues of LipB is unnecessary for its reactivation activity. This feature indicates that all of the functional residues in the reactivation exist on the region of Δ61LipB. Therefore, for the purpose of the present study, it is sufficient that only the gene of Δ61LipB is served as a target DNA in the random mutagenesis.

However, it was observed that the production of the full-length LipB (containing N-terminal 61 residues) is needed to detect the activity of LipB by the tributyrin plate assay. When the gene of the Δ61LipB instead of the full-length LipB gene was directly inserted at downstream of *lipA* in pUL11, clear zone was little detected around the colony of *E. coli* 1100 carrying this plasmid (data not shown). This result indicates that the secretion of LipA by *E. coli* cells seems to require the N-terminal region of the full-length LipB. Thus, it is relevant to employ the strategy that the gene library of Δ61LipB derived from the error-prone PCR was directly ligated to the DNA encoding the N-terminal 61 residues of pUL32. As a result, *E. coli* 1100 pUL32 produces the full-length LipB that is a mutant of K62M due to the plasmid construction. However, it was observed that this mutation does not affect the ability to form clear zone. After all, a reliable system of random mutagenesis was established that the gene of Δ61LipB is readily isolated from and ligated to the plasmids used in the present study at the common cloning sites of *Nco* I and *Hin*d III (Fig.4-1).

The random mutagenesis suggests that four residues of LipB, Asp76, Tyr99, Ser102, and Arg115 are likely to participate in its reactivation activity. The single-residue substituted mutants, Y99C, Y99H, S102R, and R115C were observed to decrease the activity of reactivating the denatured LipA, and to form no detectable complex with LipA. Notably, it is proposed that Tyr99 and Arg115 are the residues on the surface of the LipB molecule, because the single cysteine residue of Y99C and R115C formed the intermolecular disulfide bond. Thus, Tyr99 and Arg115 could directly interact with LipA. Regarding above four residues of LipB, only Tyr99 is completely conserved among the lipase activator proteins (Fig. 1-3). In the case of the activator proteins form *P. cepacia* group (Iizumi *et al.*, 1991, Jørgensen *et al.*, 1991, Nakanishi *et al.*, 1991), residues corresponding to Ser102 and Arg115 are threonine and alanine, respectively. In Chapter 2, it is demonstrated that LipB of *P. aeruginosa* TE3285 reactivated LipA from the same origin and P*seudomonas* sp. 109 lipase that has 98% identical sequence with LipA, but not from the other *Pseudomonas* species. This feature suggests that several amino acid residues of LipB would be attributed to the strict substrate specificity. In this respect, such residues important to specific recognition of LipA would not be necessarily conserved among the lipase activator proteins. Therefore, Ser102 and Arg115 of LipB may be responsible for the specificity of LipB.

CHAPTER 5

Conclusions

LipB, lipase activator protein of *Pseudomonas aeruginosa* TE3285, is a folding factor for producing active extracellular lipase (LipA) in the cell. In this study, roles of LipB in the reactivation process of LipA were analyzed in the molecular level by means of protein engineering techniques.

LipB was overproduced in *Escherichia coli*, and purified 4.9-fold over the crude extract in the presence of SDS. The purified LipB reactivated LipA that is denatured with guanidine hydrochloride, and this reactivation did not involve multiple turnover. In this reactivation, a 1:1 complex between LipA and LipB was detected in a cross-linking experiment, suggesting that LipB still binds to LipA after the reactivation. Calcium ion was essential for the complex formation and the reactivation, and addition of EDTA caused inactivation of the reactivated LipA bound to LipB more rapidly than the native LipA. These findings suggest that LipB could affect the calcium binding to LipA in the reactivation process. LipB was unable to reactivate lipases from other sources except *Pseudomonas* sp. 109; this lipase has an amino acid sequence which is 98% identical to that of LipA. Thus, it may be concluded that LipB specifically recognizes a unique structural element of LipA.

Two mutant forms, which had truncated N-terminals, of LipB were prepared, and their molecular properties and activity were compared with those of the full-length form. A truncated LipB lacking its hydrophobic N-terminal 21 residues was dispersed homogeneously in solution, and could reactivate the stoichiometric amount of denatured LipA. In contrast, full-length LipB formed soluble aggregates, and reactivated less than an equimolar amount of LipA even under the most suitable conditions. These findings suggest that some or all of the N-terminal 21 residues caused aggregation of the protein molecules, and prevented LipB from fully stoichiometric reactivation. A truncated LipB lacking the N-terminal 61 residues also re-

activated denatured LipA, suggesting that the N-terminal 61-residue region of LipB is not involved in reactivation.

To find important amino acid residues of LipB in reactivation of LipA, random mutagenesis using error-prone PCR was performed on a gene encoding Δ61LipB. The given DNA library was introduced into the lipase expression system using *E. coli*, and LipBs losing its activity was selected by two screening procedures. First, on agar plates containing tributyrin for substrate of LipA, single colonies without active LipA secretion were selected as clones missing active LipB. Second, to exclude nonsense mutants and frameshift ones, molecular size of LipB in the given clones was confirmed by Western blotting. From the selected mutants, of which multiple residues are replaced, five single-residue substituted mutants were newly prepared. Consequently, Y99C, Y99H, S102R, and R115C mutants formed no detectable complex with LipA and lost the *in vitro* reactivation activity. In the case of Y99C and R115C, their single cysteine residue formed the intermolecular disulfide bridge. Thus, Tyr99 and Arg115 are likely to exist on the molecular surface of LipB, and directly interact with the denatured LipA in the reactivation process.

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