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Calcium Ion-Dependent Reactivation of a *Pseudomonas* Lipase by Its Specific Modulating Protein, LipB[1]

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LipB, the lipase activator protein of *Pseudomonas aeruginosa* TE3285, was overproduced in *Escherichia coli*, and purified 4.9-fold over the crude extract in the presence of SDS. The purified LipB reactivated the lipase from *P. aeruginosa* TE3285 denatured with guanidine hydrochloride, and its reactivation did not involve multiple turnover. In this reactivation, a 1:1 complex between the lipase and LipB was detected in a cross-linking experiment, suggesting that LipB still binds to the lipase after the reactivation. Calcium ion was essential for the complex formation and the reactivation, and addition of EDTA caused inactivation of the reactivated lipase bound to LipB more rapidly than the native lipase. These findings suggest that LipB could affect the calcium binding to the lipase 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 the lipase from *P. aeruginosa* TE3285. Thus, it may be concluded that LipB specifically recognizes a unique structural element of the lipase.

Key words: calcium ion, complex, lipase activator protein, *Pseudomonas*, reactivation.

Protein folding is one of the most important biological processes, and various folding-mediating factors control this process in the cell. A ubiquitous group of them, called molecular chaperone, acts on a wide variety of non-native polypeptides during post-translational events (*1*). However, it has been found that several species of *Pseudomonas* produce another type of folding factor proposed to act specifically on their extracellular lipase [EC 3.1.1.3] (*2, 3*).

We have cloned the genes of lipase (LipA; molecular weight of 30,100) and its activator protein, LipB (molecular weight of 37,500), from *P. aeruginosa* TE3285, and have demonstrated that the *lipB* gene is essential for the production of the active LipA *in vivo* (*4*). *In vitro* experiments have indicated that a fusion protein of glutathione transferase-LipB activates both LipA solubilized from the inclusion bodies overproduced in *E. coli* and LipA inactivated by denaturation (*5*). Therefore, LipB has been thought to possess a chaperone-like activity to fold LipA into the active conformation. The same types of lipase activator proteins were also found from other *Pseudomonas* species (*6-10*) and *Acinetobacter* (*11*). In any case, the gene of the activator protein forms a bicistronic operon with the gene of the lipase. The *lipB* gene is located downstream of the structural gene of LipA, and these genes are considered to form a single operon based on the gene structure. That is, the folding factor is encoded at a position adjacent to the gene of its substrate protein. In this respect, the lipase activator protein seems to be similar to the N-terminal propeptide of several proteases such as subtilisin, α-lytic protease, and carboxypeptidase Y (*12-14*). These propeptides are produced as a single polypeptide with its target protease, and specifically assist its folding.

Calcium ion is an important factor in the propeptide-mediated folding of subtilisin. It has been considered that calcium binding to subtilisin in the folding process is regulated by its propeptide (*15*). 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 (*16-19*). Thus, it could be presumed that the lipase activator protein participates in this calcium binding during the reactivation process of lipase.

In the present study, we purified the recombinant full-length LipB and kinetically analyzed its function *in vitro*. The purified LipB reactivated denatured LipA, but did not dissociate from the reactivated LipA *in vitro*. We also investigated the effect of calcium ion on the LipB-assisted reactivation, and we propose that LipB specifically assists on the binding of calcium ion to LipA.

EXPERIMENTAL PROCEDURES

Materials—Bacterial strain *Escherichia coli* BL21(DE3) and plasmid pET-3d (Novagen) were used as a host and a vector (*20*) for expression of *lipB*, respectively. Plasmid pUL11 is a pUC19 derivative carrying both *lipA* and *lipB* from *Pseudomonas aeruginosa* TE3285 (*4*). Synthetic oligonucleotide was a gift from Toyobo (Osaka). Restriction

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enzymes and DNA modifying enzymes were obtained from Toyobo and Takara Shuzo (Kyoto). The LipA protein purified from culture medium of *P. aeruginosa* TE3285 (*4*) was a special gift from Toyobo. The purified lipases from *Pseudomonas* sp. 109 (*21*) and *P. cepacia* M-12-33 were generous gifts from Nagase Biochemicals (Tokyo) and Amano Pharmaceutical (Nagoya), respectively. The purified lipases from *Candida cylindracea* and porcine pancreas were obtained from Sigma Chemical. Cellulofine GCL-1000m was a gift from Chisso (Tokyo). 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 follows. A *Kpn*I-*Bam*HI fragment of pUL11 containing *lipA* and *lipB* was inserted into M13mp19. An *Nco*I site was introduced at the position of the initiation codon of *lipB* by site-directed mutagenesis according to the method of Kunkel (*22*). The mutational primer consists of the sequence 5′-GCCCCCTCCCATGGG-GAAAATCCTCCTGC-3′, where the mismatched bases are underlined. The plasmid pELB10 was prepared by inserting an *Nco*I-*Hin*dIII fragment including *lipB* at the position downstream of the T7 promoter in pET-3d.

Production 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 used 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). The active fractions including 1.5 mg proteins were applied to SDS polyacrylamide gels (180×180×2 mm) and electrophoresed as described by Laemmli (*23*) at 20°C. The gels were stained with Copper Stain (Bio-Rad Laboratories) (*24*), and a band including LipB was excised from the gels. The purified protein was recovered by electroelution (*25*). The eluate was applied to an Extracti-Gel D column (Pierce Chemical) 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) (*26*). Bovine serum albumin was used as a standard. Lipase activity was measured with Lipase Kit S (Dainippon Pharmaceutical, Osaka), in which 2,3-dimercaptopropan-1-ol tributyrate was used as a substrate (*27*). One unit (U) was defined as an amount of lipase catalyzing the hydrolysis of 1 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 the mixture 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 (*28*). The reaction was then quenched by adding 40 μl of 2 M sodium borohydride dissolved in 0.1 M NaOH. Protein mixtures were precipitated with 7% trichloroacetic acid (*29*), and analyzed by SDS-PAGE (*23*).

RESULTS

Overexpression of lipB in E. coli—LipB was overproduced by using the T7 expression system (*20*). An expression plasmid for *lipB*, pELB10, was constructed by inserting *lipB* at the position downstream of the T7 promoter in pET-3d. 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. 1, 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* (37.5 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* (*4*) 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 I). The result of SDS-PAGE analysis of each purification step is shown in Fig. 1.

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—The denatured LipA gradually recovered its lipase activity in the presence of LipB, whereas no lipase activity was recovered without LipB (Fig. 2). 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/liter, 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* (*5*) was used for a substrate of LipB (data not shown). In addition, we 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.

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. 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

TABLE I. **Purification of the recombinant full-length LipB.** The specific activity of LipB was determined from the initial rate of reactivation of the 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

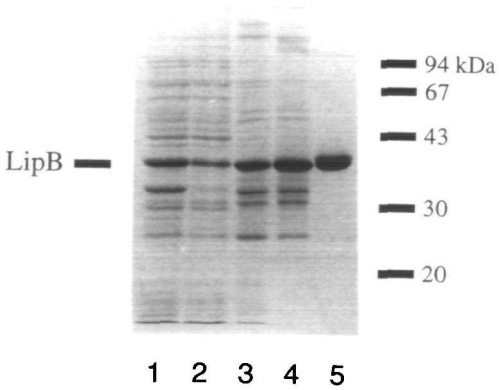

Fig. 1. **SDS-PAGE analysis of the purification of LipB.** A 12.5% polyacrylamide gel was used, and proteins were stained with Coomassie Brilliant Blue R-250. Lane 1, total cells; lane 2, crude extract; lane 3, ammonium sulfate fraction; lane 4, Cellulofine GCL-1000m eluate; lane 5, preparative SDS-PAGE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

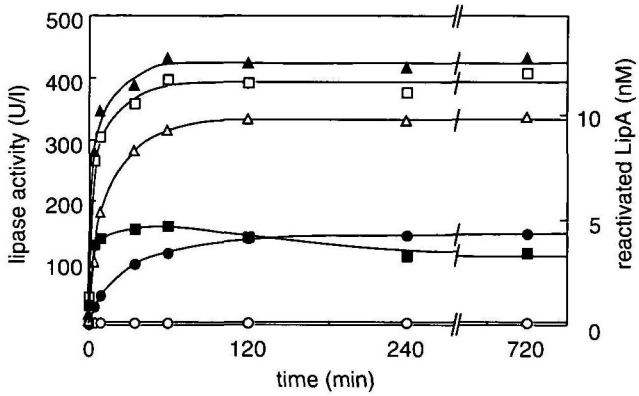

Fig. 2. **Time course of the LipB-assisted reactivation of the denatured LipA.** The denatured LipA solution was diluted 41-fold into LipB solution and incubated at 20°C. The final concentration of the denatured LipA was 0.29 μM. The concentrations of LipB were as follows: 0.0 nM (○); 11 nM (●); 56 nM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

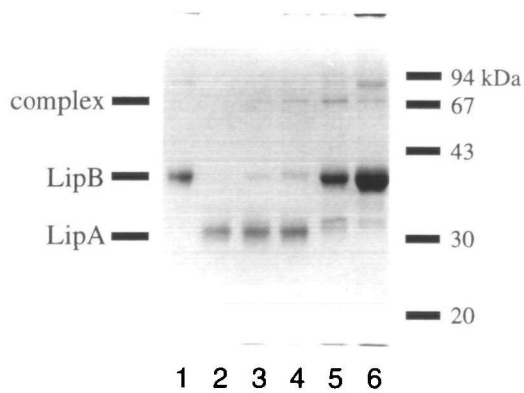

Fig. 3. **SDS-PAGE analysis of the LipA-LipB complex cross-linked with glutaraldehyde.** Protein mixtures on reactivation were treated with glutaraldehyde. A 12.5% polyacrylamide gel was used, and proteins were stained with Coomassie Brilliant Blue R-250. Lane 1, LipB; lane 2, the denatured LipA; lanes 3-6, LipB and the 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, 1,400 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.

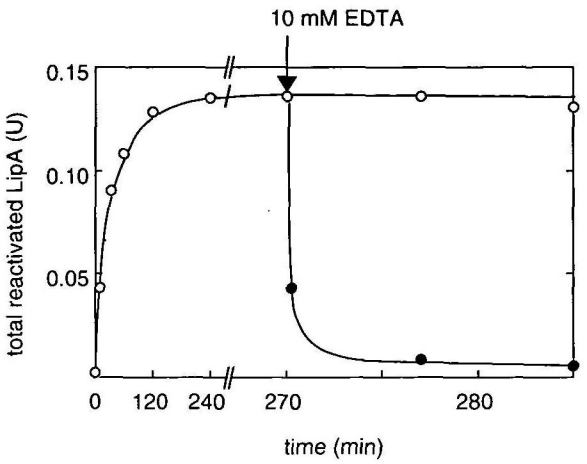

Fig. 4. **Inactivation of the reactivated LipA by addition of EDTA.** The reactivation was started by addition of 25 μl of the denatured LipA solution to 1 ml of 6 nM LipB solution at 20°C under the standard condition. EDTA solution (final 10 mM) was added at 270 min from the start of the reactivation. Total lipase activity units are plotted for the reactivation mixture with EDTA (●) and without EDTA (○).

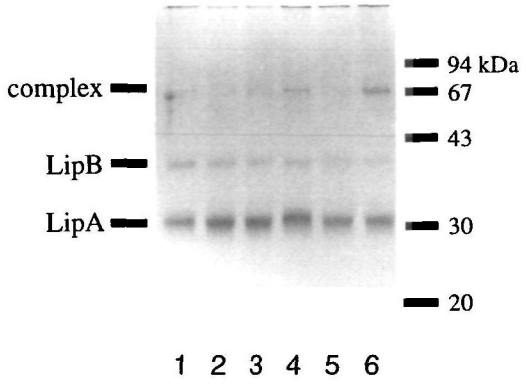

Fig. 5. **SDS-PAGE analysis of the effect of calcium ion on the LipA-LipB complex.** A 12.5% polyacrylamide gel was used, and proteins were stained with Coomassie Brilliant Blue R-250. The reactivation buffer contained Triton X-100 at the concentration of 0.5% (lanes 1-3) or 0.05% (lanes 4-6). For lanes 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. The protein mixtures were cross-linked with glutaraldehyde as described in "EXPERIMENTAL PROCEDURES".

solution for cross-linking. The products were analyzed by SDS-PAGE (Fig. 3). A 68-kDa protein was newly observed (lanes 3-6), corresponding in size to the sum of LipA (30.1 kDa) and LipB (37.5 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). 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). Thus, it is suggested that this complex detected by cross-linking expresses the lipase activity recovered with LipB.

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 $MgCl_2$, 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. 4). 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. We therefore analyzed the effect of calcium ion on the complex formation between LipB and LipA in the reactivation by cross-linking (Fig. 5). Without $CaCl_2$ the cross-linked band was not clearly detected (lane 2), indicating that calcium ion also affected the complex formation between 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 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 the LipA-LipB complex formation was not clearly detected (Fig. 5, 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 (Fig. 5, lane 6).

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

DISCUSSION

We have purified the full-length LipB of *P. aeruginosa* TE3285, and the isolation of the purified LipB allowed us to analyze 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, we have examined the effects of several coenzymes on the LipB-assisted reactivation of the denatured LipA. 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 (*30*). Secretion of *Pseudomonas* lipase is also proposed to be mediated by Xcp proteins (*31*). Furthermore, it is observed that the lipase activator protein is located in periplasm (*10, 32*). Thus, one or more Xcp proteins are candidate for direct participation in the dissociation of the complex between LipB and the active LipA in periplasm.

We have 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 (*33*). The calcium binding site of other bacterial lipases was determined by crystal structure analyses (*16-19*). 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, we suggest 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 (*15*).

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.* (*6*) suggested that lipase activator proteins function as a private chaperone for the *Pseudomonas* lipases based on their gene construction. Our 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 (*32*). 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 (*10*). 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.

In conclusion, we have demonstrated that calcium ion is required for the LipB-assisted reactivation of denatured LipA. LipB has been proposed to play a specific role in the calcium binding to LipA. After the reactivation, LipB is considered still to form a productive complex with the reactivated LipA, and an additional unknown factor would be necessary to release the active LipA from LipB. We have indicated that the substrate specificity of LipB is restricted to LipA, and the N-terminal region of LipB is related to the requirement of a detergent in LipB purification and LipA reactivation.

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