Articles

Identification of a Calcium Binding Site in *Staphylococcus hyicus* Lipase: Generation of Calcium-Independent Variants†

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ABSTRACT: In this study we have identified the presence of a high-affinity binding site for calcium in the lipase from *Staphylococcus hyicus*. By means of isothermal titration calorimetry we showed that the enzyme binds one calcium per molecule of enzyme with a dissociation constant of 55 μM. The residual activity of the apoenzyme compared to the activity in the presence of calcium ions varies from 65% at 10 °C to nearly zero at 40 °C. On the basis of primary sequence alignment with other staphylococcal lipases and the lipases from *Bacillus thermocatenulatus* and from *Pseudomonas glumae* in combination with site-directed mutagenesis, aspartates 354 and 357 could be identified as calcium ligands. Kinetic measurements with the D357E variant showed that replacement of Asp357 by a glutamate decreased the affinity for calcium ions 30-fold. Introduction of a lysine, an asparagine, or an alanine at position 357 and of a lysine or an asparagine at position 354 resulted in calcium-independent variants. Isothermal titration calorimetry confirmed the loss of calcium binding. Although the D357K, D357N, and D357A variants did not bind calcium, at room temperature they were nearly as active as wild-type lipase in the presence of calcium, but at elevated temperatures these calcium-independent lipases showed a reduced activity. Over the whole temperature range the activities of the D354K and D354N variants are significantly lower than wild-type enzyme in the presence of calcium and are comparable to the activity of the wild-type apoenzyme. Our results show that binding of calcium is important for the structural stabilization of staphylococcal lipases (and possibly other lipases) and that it is possible to engineer calcium-independent variants on the basis of limited structural homology with another lipase.

Lipases (glycerol ester hydrolases, EC 3.1.1.3) are versatile enzymes that have been isolated from a variety of eukaryotic and prokaryotic organisms. Lipases hydrolyze the ester bonds in long-chain triacylglycerols, and in addition to these natural substrates they hydrolyze a wide range of other substrates including synthetic lipids. Moreover, it has been reported that lipases often show enantio-, regio-, and chain length selectivities (ref *1* and references therein). In addition, these enzymes are very stable in organic solvents, where they catalyze (trans)esterification reactions (*2*, *3*). Because of these properties, lipases are suitable enzymes for industrial applications, which certainly has contributed to the increased interest in these enzymes over the past decade.

After the first X-ray structures of human pancreatic lipase and *Rhizomucor miehei* lipase became available (*4*, *5*), more than 20 lipase structures have been determined. As a result our understanding of the functioning of these enzymes has been substantially improved. Although the overall homology of lipases is low and molecular masses vary from 20 to 60 kDa, all lipases share a comparable three-dimensional fold, which is known as the α/β hydrolase fold (*6*). Apart from this highly conserved fold of the central core, diversity among lipases mainly results from extrastructural extensions displaying low homology. The region of highest conservation is the active site, which contains a "classical" Ser-His-Asp catalytic triad, and residues involved in the oxyanion hole. Interestingly, in most lipase structures the active site is inaccessible due to coverage by one or more surface loops or helical structures. The structures of lipases bound to (substrate) micelles (*7*, *8*) and of lipases inhibited by transition-state analogues (*9*, *10*) showed that the active site becomes exposed to substrate upon interaction with micelles or substrate molecules. These studies provided a structural

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basis for the well-known phenomenon of "interfacial activation" as it was discovered nearly 40 years ago (*11*).

The catalytic reaction involves a nucleophilic attack of the active-site serine on the cleavable ester bond in the substrate molecule. This reaction results in the release of an alcohol moiety and the formation of an acyl-enzyme intermediate. This intermediate is susceptible to a second nucleophilic attack by a water molecule, which releases the fatty acid and regenerates active enzyme. For several lipases an enhancement of this reaction in the presence of calcium ions has been reported, but the mechanism of this activation was not well understood (*12*−*14*). A possible clue came when Noble and co-workers solved the crystal structure of the lipase from *Pseudomonas glumae* (PGL), revealing the presence of a calcium ion bound to the protein (*15*). Because of the distance between this calcium binding site and the active site, a direct involvement of calcium in catalysis seemed unlikely, and therefore the authors proposed a structural role for this metal ion in PGL.

We recently reported the purification and characterization of the lipases from *Staphylococcus aureus* (SAL1), *Staphylococcus epidermidis* (SEL), and *Staphylococcus hyicus* (SHL) (*16*, *17*). These lipases have a low, but significant, residual activity of about 5% in the absence of calcium and only become fully active upon addition of saturating calcium concentrations. Moreover, we showed that calcium protects these proteins against urea-induced unfolding (*18*). These observations suggest that calcium ions activate these lipases by a structural stabilization, but thus far no conclusive evidence for this hypothesis has been obtained. In the present study we used isothermal titration calorimetry (ITC) in combination with site-directed mutagenesis to further characterize the importance of calcium for SHL activity. The identification of two aspartates that are involved in calcium binding allowed us to construct SHL variants that do not bind calcium and that show calcium-independent activity.

MATERIALS AND METHODS

Chemicals. Triton X-100 (TX100) was obtained from Serva and tributyrylglycerol was from Aldrich. *p*-Nitrophenyl butyrate (PNPB) was purchased from Sigma. Small-scale gel-filtration PD-10 columns (Sephadex G-25) were purchased from Pharmacia.

Bacterial Strains, Plasmids, and DNA Techniques. *Escherichia coli* strains were grown in Luria−Bertani medium, supplemented with 100 μg/mL ampicillin for plasmid maintenance. The expression plasmid pH_6SHT_7 contains the structural gene for mature SHL (*19*), fused to an N-terminal hexahistidine tag, under control of the promotor for T7 RNA polymerase (*16*). Lipases were expressed in strain Bl21-(DE3), which carries the structural gene for T7 RNA polymerase under control of an IPTG-inducible promoter (*20*). Plasmid pUC18 and strain DH5α (*21*) were used for the introduction of mutations. Competent bacterial cells were transformed with plasmid DNA by using the $CaCl_2$ method (*22*). Plasmid DNA was isolated by using plasmid mini- or midiprep isolation kits (Qiagen). Synthetic oligonucleotides were obtained from Pharmacia. Restriction endonucleases were from New England Biolabs. DNA fragments were isolated from an agarose gel by using the Qiaex DNA gel extraction kit (Qiagen). DNA sequencing was performed on double-stranded template DNA by the dideoxy chain-termination method (*23*) with the T7 DNA polymerase sequencing kit (Pharmacia).

Site-Directed Mutagenesis. The lipase-encoding *Xba*I−*Pst*I fragment of pH_6SHT_7 was subcloned into pUC18 by using the corresponding sites of the polylinker. Mutations were introduced by a PCR-based method, essentially as described before (*24*). For screening purposes, in each set of mutagenic primers an endonuclease restriction site was introduced simultaneously with the mutation or at another site by silent mutagenesis. The following set of primers was used (only the 5′ → 3′ strands of each mutagenic primer couple are given, with the mutation site in boldface type and the diagnostic restriction site in italic type) 1 (D210N, *Ase*I), CATTTAGGAACG*ATT**AAT***TTTGGCATGGAC; 2 (E224Q, *Hin*dIII), CGTAAAGATGGT**C*A**AAGCTT***AACCGATTATAATAAG; 3 (D243N; *Stu*I), GGATTCTGAA**AAC**AC*AGG**CCT***TTATGATTTAAC; 4 (D248N, *Mlu*I), CAGGGCTTTAT**AAC**TTA*AC**C**CGT*GAAGGAG; 5 (E252Q, *Nar*I), GATTTAACGCGT**CAG***GG**C**GCC*GAAAAAATTAATC; 6 (D288N; *Avr*II), GCAAACATATCGCT**A**A*CCTAGG*TATGGAATTC; 7 (D357E, *Eco*RI), GGTTGGGATCATAGT*GA**ATTC***ATTGGAAATGATGC; 8 (D357K, *Apo*I), GGTTGGGATCATAGT***AAATTT***ATTGGAAATGATGC; 9 (D357N, *Bst*BI), GGTTGGGATCA***TTCGAA***CTTTATTGGAAATGATGC; 10 (D357A, *Eco*47III), GGTTGGGATCATA*G**CG**CT*TTTATTGGAAATG; 11 (D354N, *Dde*I), CATGAAAGGTTG-G**A**ACCA*CTCAG*ACTTTATTGG; 12 (D354K, *Dde*I), CATGAAAGGTTGG**A**A**A**CA*CTCAG*ACTTTATTGG.

PCRs were performed in 0.5 mL Eppendorf tubes with a Techne programmable Ori-block PHC-1. Template DNA (25 ng) was mixed with 125 ng of both mutagenic primers in 50 μL of polymerase reaction buffer containing 50 μM of each deoxynucleoside triphosphate. After addition of 2.5 units of cloned *Pfu* DNA polymerase (Stratagene), the reaction mixture was overlayered with 30 μL of mineral oil. The thermal profile included a first denaturating step at 95 °C for 2 min followed by 18 cycles of denaturating at 95 °C for 30 s, primer annealing at 55 °C for 1 min, and extension at 68 °C for 8 min. After this cycling the reaction mixture was cooled at 4 °C for 2 min. Subsequently, 20 units of *Dpn*I was added, followed by incubation at 37 °C for 1 h. The *Dpn*I-restricted PCR mixture was transformed to competent *E. coli* DH5α cells. After plasmid isolation, clones containing mutated DNA were identified by restriction analysis. Subsequently, the DNA sequences of fragments *Msc*I−*Mlu*I (D210N, E224Q, and D243N), *Msc*I−*Mfe*I (D248N and E252Q), *Mfe*I−*Eco*RI (D288N), and *Eco*RI−*Pst*I (D354N, D354K, D357E, D357K, D357N, and D357A) were verified by sequencing. DNA fragments that contained the mutations were subcloned into the expression vector pH_6SHT_7 by exchange of the corresponding fragments.

[1] Abbreviations: BTL, *Bacillus thermocatenulatus* lipase; PGL, *Pseudomonas glumae* lipase; SAL1, *Staphylococcus aureus* lipase from strain NCTC8530; SAL2, *Staphylococcus aureus* lipase from strain PS54; SEL, *Staphylococcus epidermidis* lipase; SHL, *Staphylococcus hyicus* lipase; ITC, isothermal titration calorimetry; IPTG, isopropyl thio-β-D-galactopyranoside; TX100, Triton X-100; PNPB, *p*-nitrophenyl butyrate; EDTA, *N*,*N*,*N*′,*N*′-ethylenediaminetetraacetic acid; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; WT, wild type.

Small-Scale Expression of SHL Variants. To determine the activity of variants in crude cell lysates of *E. coli* Bl21-(DE3), an overnight culture of cells containing the desired expression plasmid was diluted 20-fold in 10 mL of LB medium. Incubation took place at 37 °C under vigorous stirring and after 1.5 h of growth, lipase production was induced by the addition of IPTG at a concentration of 0.4 mM. After another 4 h of growth the cells were collected by centrifugation (20 min, 5000*g*, 4 °C). The cell pellet was dissolved in 500 μL of 8 M urea and lipase activity was determined by diluting 10 μL of this solution into the pH-stat activity assay.

Purification of SHL Variants. His-tagged SHL variants were produced on a 1 L scale in *E. coli* Bl21(DE3), containing the corresponding pH_6SHT_7 derivative, as described above for small-scale cultures. The purification of the lipases was done at 4 °C as described for His-tagged WT-SHL (*16*). The purity of the variants was checked by SDS−PAGE analysis on 15% acrylamide gels, which in all cases showed one single band of protein. Gels were stained with Coomassie Brilliant Blue (*22*). Lipase concentrations were determined by absorbency using an $OD_{280}^{1\%}$ value of 14.5. Typical yields were 10−15 mg of pure lipase.

Lipase Activity Assays. Enzymatic activities were determined in a pH-stat assay by titrating released fatty acids with 5 mM sodium hydroxide under nitrogen using a Radiometer titration set consisting of a PHM-84 pH meter, a TTT-80 titrator, an ABU-80 autoburet, a TTT-60 titration assembly, and a Rec-80 servograph. Mixed micelles of tributyrylglycerol (60 mM) with TX100 (120 mM) were used as a substrate in a buffer composed of 5 mM Tris-HCl (pH 8), 50 mM NaCl, and either 10 mM $CaCl_2$ or 5 mM EDTA. The added amount of enzyme varied between 0.5 and 20 μg, depending on the variant used and the reaction conditions. Unless stated otherwise the reaction temperature was 40 °C. The calcium dependence of purified enzymes was determined kinetically (*16*).

In a spectrophotometric assay, activities with PNPB were determined at 18 °C by following the increase in absorbance at 400 nm due to the release of the *p*-nitrophenol anion. Activities were calculated from the slopes of the reaction progress curves using a molar extinction coefficient of 18 577 M^{-1} cm^{-1}. The reaction mixture contained 1 mM PNPB, 10 mM Triton X-100, 50 mM Tris-HCl (pH 8), 50 mM NaCl, and either 10 mM $CaCl_2$ or 5 mM EDTA.

Isothermal Titration Calorimetry. ITC measurements were performed with a Microcal MCS titration calorimeter. Protein solutions were prepared by dissolving approximately 10 mg (0.22 μmol) of lyophilized SHL in 2.5 mL of buffer A composed of 50 mM Tris-HCl (pH 8) and 100 mM KCl. This solution was applied to a PD-10 column equilibrated in the same buffer. The eluted protein was subjected to the same procedure once again, and subsequently the SHL concentration was determined by absorbency. A volume of 1.3 mL of this solution was introduced into the cell of the calorimeter. The titration was performed by injecting 5 μL portions of buffer A, containing in addition 2 mM $CaCl_2$, at a temperature of 40 °C. The response time for the first 10 additions was set to 800 s and for the residual 35 additions it was 400 s. After subtraction of the baseline measured in the absence of protein, the binding constants and the number of bound Ca^{2+} ions were calculated as described before (*25*) by using the computer program ORIGIN (Microcal, Northhampton, MA).

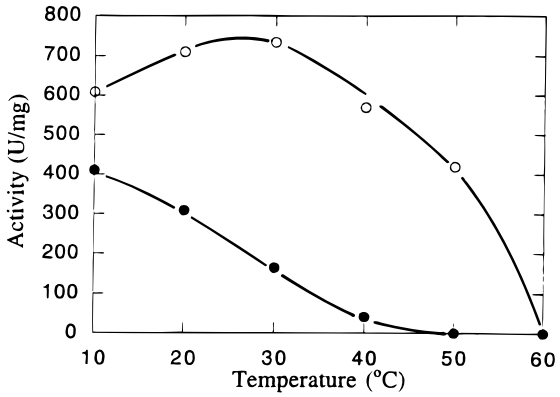

FIGURE 1: Temperature profiles for the activity of WT-SHL in the presence and absence of calcium. Mixed micelles of tributyrylglycerol (60 mM) and TX100 (120 mM) were used as a substrate, and reactions were performed in a buffer composed of 5 mM Tris-HCl (pH 8) and 50 mM NaCl. In addition, either 10 mM $CaCl_2$ (○) or 5 mM EDTA (●) was present.

Sequence Alignment of Lipases. First the sequences of SHL, SAL1, SAL2, SEL, and BTL were aligned using the alignment option in the MacVector package (Oxford Molecular Group). Attempts to align PGL with the same procedure failed because homology is low and moreover PGL is 67−80 amino acids smaller than the staphylococcal lipases. Therefore, the PGL sequence was aligned by hand by fixing the positions of the active-site residues Ser, Asp, and His and of Leu17, which contributes to the oxyanion hole in PGL. Gaps were initially introduced randomly, but leaving intact β-strands and α-helices as observed in the X-ray structure of PGL were left intact (*15*). In a next step secondary structure predictions of the SHL, SAL1, SAL2, SEL, and BTL sequences were done with the programs Predator (EMBL Facility, Heidelberg, Germany), and a combination of Chou−Fasman and Robson−Garnier. When both methods yielded similar predictions, and when at least three of these five sequences yielded the same prediction, a positive score was assigned. Finally, the alignment was optimized by hand by positioning of common structural elements of the staphylococcal lipases, BTL, and PGL and by readjusting gaps.

RESULTS

Previously the residual activity of SHL in the absence of calcium was reported to be only 5% by a pH-stat assay (*16*, *26*). To our surprise, SHL showed approximately 50% residual activity by a chromogenic assay. Since the pH-stat assay was performed at 40 °C and the spectrophotometric assay at 18 °C, we decided to investigate the relation between calcium dependence and reaction temperature more closely. To this end, the activity of SHL was determined at temperatures between 10 and 60 °C, either in the presence of saturating calcium concentrations (10 mM) or in the absence of calcium ions (5 mM EDTA). The resulting temperature profiles are shown in Figure 1 and it is clear that the calcium dependence of SHL is strongly influenced by the reaction temperature. Although only a 2.5-fold activation by calcium was observed at room temperature, the requirement for calcium becomes virtually absolute at elevated temperatures.

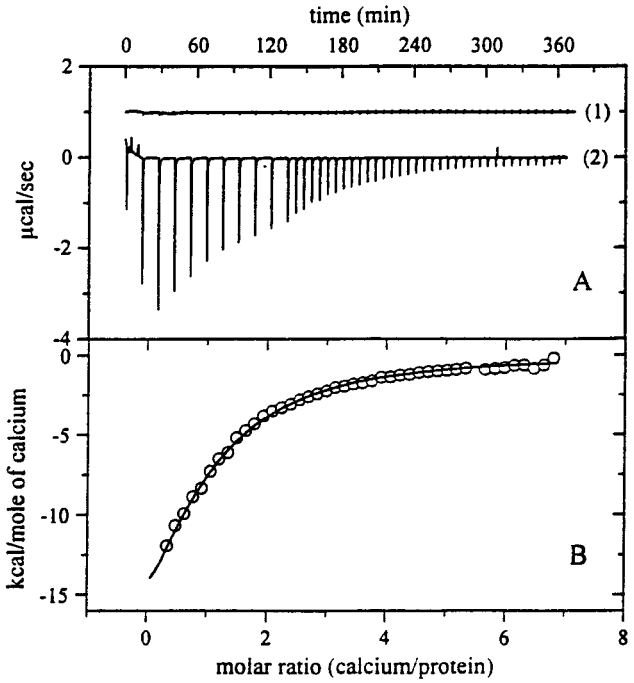

FIGURE 2: Isothermal titration curves of WT-SHL apoenzyme with calcium at 40 °C. Panel A shows the observed heat signals obtained for 45 automatic injections, each of 5 μL, of a 2 mM $CaCl_2$ solution into the sample cell containing either buffer only (1) or a solution of 52.5 μM SHL in buffer (2). The buffer was composed of 50 mM Tris-HCl (pH 8) and 100 mM KCl. Panel B shows integrated injection heats corrected for heats of calcium dilution. The molar ratio represents the concentration of added calcium over protein present in the cell. The K_d and the stoichiometry were determined from the experimental data according to a simple noncooperative model (*25*).

This observation suggests that calcium ions stabilize the active conformation of SHL, in particular at higher temperatures where the enzyme is more susceptible to denaturation.

To characterize this calcium binding, we initially used fluorescence spectroscopy. It appeared, however, that SHL fluorescence is not sensitive to calcium binding, and therefore we decided to use isothermal titration calorimetry (ITC) instead. The results of a typical ITC titration experiment of SHL apoenzyme with calcium at 40 °C are shown in Figure 2. In panel A the experimental data are given, and in panel B the data are processed as described by Wiseman and coworkers (*25*). The data points in the latter curve correspond to the negative heat of calcium binding associated with each addition of calcium. The K_d and the stoichiometry were determined from these data according to a simple noncooperative model (*25*). Fitting of two independent titration curves yielded an average stoichiometry of 1.09 ± 0.2 calcium ions per SHL molecule and a calcium dissociation constant of 55 ± 5 μM. Thus, apparently SHL has a single, high-affinity calcium binding site.

No X-ray structure of a staphylococcal lipase is available, but interestingly, in the X-ray structure of another bacterial lipase from *Pseudomonas glumae* (PGL), a calcium binding site has been identified (*15*). Therefore, we used a primary sequence alignment of the staphylococcal lipases and PGL to search for putative calcium binding residues in SHL (for details of the alignment procedure, see the Materials and Methods section). We also included the sequence of *Bacillus thermocatenulatus* lipase (BTL), which is a thermophilic lipase homologous to the staphylococcal lipases (*27*). Figure 3 shows the alignment of SHL with the sequences of SAL1, SAL2, SEL, BTL, and PGL. It can readily be seen that the staphylococcal lipases and BTL are closely related, sharing about 35% identical amino acids, whereas an additional 35% represents structurally and functionally related amino acids. Previous characterization has shown that the activity of SHL, SAL1, and SEL is enhanced by calcium ions (*16*, *17*). BTL is of interest because we were able to show that at 60 °C the activity of this thermostable enzyme is enhanced 3-fold by the presence of calcium ions (data not shown). In this respect BTL behaves at 60 °C like SHL at 25 °C (see previous section). Since the binding of calcium by proteins mostly involves backbone carbonyl oxygens and aspartate or glutamate side chains (*28*), all 28 acidic residues (excluding the active-site Asp) that are conserved in SHL, SAL1, and SEL are putative candidates for calcium binding. When the alignment of SAL2, which is less well characterized, was included as well, this number reduced to 20. It is of interest to note that 13 of these residues are conserved in BTL.

To further limit the number of putative calcium ligands, the alignment of PGL was included as well. In PGL the calcium ion is fixed between the negative charges of Asp241 and Asp287, and in addition two backbone carbonyl oxygens contribute to binding (*15*). As is obvious from Figure 3, PGL is less related to the staphylococcal lipases than they are among each other, and homology is most easily recognized in the regions near the active-site residues Ser, Asp, and His. One of the calcium binding residues of PGL (Asp287) is located in a conserved region surrounding the active-site histidine. Interestingly, at this position an acidic residue is conserved among all aligned lipases. The second calcium ligand of PGL (Asp241) is located in a surface loop between the α-helices 8 and 9 preceding the active-site Asp, and again an acidic residue is found for all lipases.

First, Asp357 of SHL, which was most easily identified as the equivalent of Asp287 in PGL involved in calcium binding, was replaced by a glutamate. It was expected that this subtle change reduces the affinity for calcium without disturbing possibly important interactions for the active-site geometry. The cell lysate of a small-scale culture was checked for the expression of the variant enzyme by SDS−PAGE analysis. The variant was produced at a level comparable to WT-SHL, i.e., 4 h after induction with IPTG, an estimated amount of 100−200 μg of both enzymes per 10 mL of culture was present. The enzymatic activities in the crude cell lysates of three independently grown cultures were determined with tributyrylglycerol as the substrate. At 40 °C in the presence of 10 mM $CaCl_2$, lysates of cells carrying the variant D357E-SHL had an activity of about 70% compared to WT-SHL. To determine the precise specific activity and the affinity for calcium ions of D357E-SHL, we purified this variant lipase to homogeneity. At 40 °C and in the absence of calcium, D357E-SHL had a low activity of 43 units/mg. At saturating calcium levels the activity increased to a value of 470 units/mg, comparable to the 570 units/mg for WT-SHL. When the calcium concentration was varied, saturation curves were obtained that are depicted in Figure 4. From these curves calcium dissociation constants of 450 μM for D357E-SHL and of 16 μM for WT-SHL were calculated. This 30-fold decrease in calcium affinity as a result of this single amino acid substitution

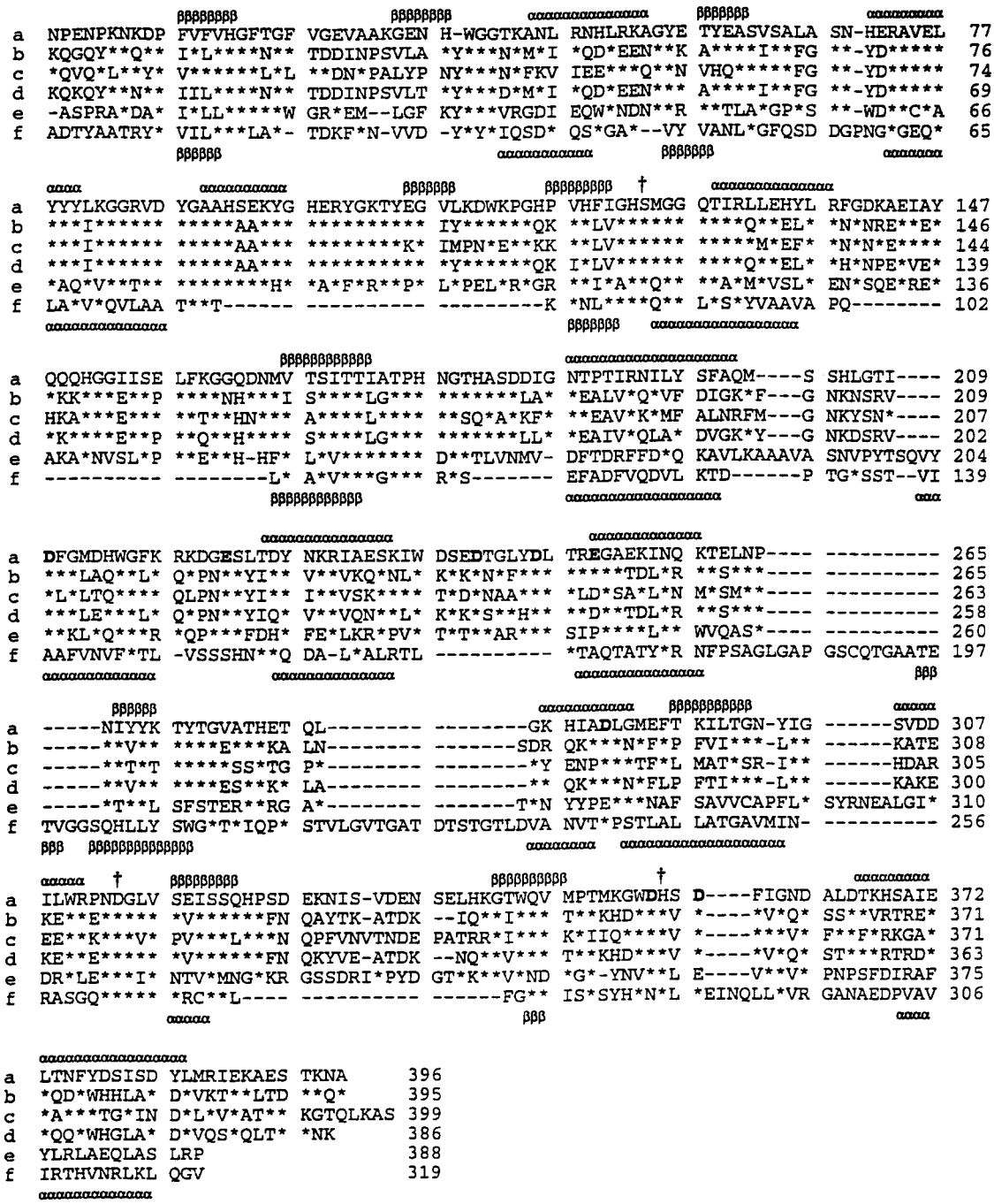

FIGURE 3: Primary sequence alignment of the six bacterial lipases. Shown are the sequences of the lipases from *S. hyicus* (a), *S. aureus* NCTC8530 (b), *S. aureus* PS54 (c), *S. epidermidis* RP62A (d), *B. thermocatenulatus* (e), and *P. glumae* (f). Conserved residues are indicated by asterisks. The active-site residues are indicated by daggers (†), and amino acids chosen to identify the second calcium ligand in SHL are given in boldface type. Helices (α) and sheets (β) identified in the X-ray structure of PGL are given below the sequences, and predictions for the secondary structure elements of staphylococcal and *B. thermocatenulatus* lipases are given above the sequences.

strongly suggests that residue Asp357 of SHL is indeed involved in calcium binding.

Although we identified only one calcium ligand in SHL, we assumed that the calcium binding site of SHL contains two acidic side chains, like in PGL. On the basis of this assumption, we anticipated that the presence of a lysine residue might provide sufficient positive charge density to stabilize the SHL structure in the absence of calcium ions. To verify this hypothesis, a mutation coding for an Asp to Lys substitution at position 357 was introduced into the SHL gene. After purification, the activity of D357K-SHL was determined at 40 °C. From the data presented in Table 1 it is clear that this Asp to Lys mutation reduces the absolute activity about 4-fold and, more importantly, that the activity remains high in the absence of calcium ions. The absence of a heat signal in ITC experiments indicated that the calcium-independent activity of this protein is coupled to the disappearance of calcium binding.

To verify that the presence of a positive charge, either from calcium or from the lysine residue, is essential for enzymatic activity, we constructed the variants D357N and D357A. Both with the isosteric replacement of aspartate by asparagine and with the nonisosteric aspartate to alanine substitution, we expected a loss of calcium binding. Indeed,

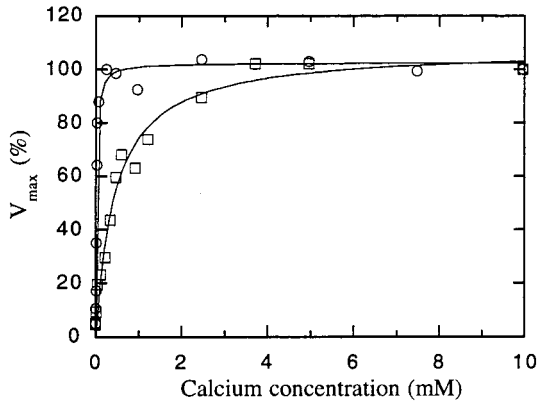

FIGURE 4: Kinetically determined calcium binding curves of D357E-SHL (□) and WT-SHL (○). The composition of the substrate solution was as described in the legend of Figure 1. Activities were determined at 40 °C. Under these conditions the maximum activities of WT-SHL and D357E-SHL were 570 and 470 units/mg, respectively.

Table 1: Enzymatic Activities of WT and Variant SHLs in the Absence and Presence of Calcium Ions

	activity[a] (units/mg)	
SHL	5 mM EDTA	10 mM $CaCl_2$
WT	28	570
D357E	43	470
D357K	138	167
D357N	130	158
D357A	173	167
D354N	56	80
D354K	14	16

[a] Activities were determined with purified enzymes by using a pH-stat assay at 40 °C as described in Materials and Methods. Activities given are average values of duplicate measurements where the accuracy is about 10%.

after purification of the variant enzymes, this lack of calcium binding was found by ITC experiments, since no significant heat signal was obtained upon addition of calcium ions (data not shown). Surprisingly, the activity of these variants was comparable to that of D357K-SHL, both in the absence and in the presence of calcium ions (Table 1). The observation that D357A behaves like D357N excludes the possibility that the asparagine stabilizes the structure of SHL by forming a hydrogen bond with the yet-unidentified second acidic residue. Taken together our results suggest that neither the presence of a positive charge nor the presence of a hydrogen-bond donor or acceptor is required for stability, but that just the absence of the negative charge is sufficient for calcium-independent activity.

In an attempt to localize the second calcium ligand we made the variant D288N. In SHL Asp288 occupies the equivalent position of the second calcium ligand (Asp241) in PGL (Figure 3). At 40 °C the purified variant had full wild-type activity in the presence of calcium, whereas its residual activity in the absence of calcium ions was around 5%, and we concluded that Asp288 in SHL is not involved in calcium binding. In this region the homology between PGL and the other lipases is low, which might give rise to an uncertainty in the alignment explaining the result obtained with the D288N variant. Therefore, we replaced in this region the acidic residues conserved in the staphylococcal enzymes with the corresponding amides. In SHL the aspartates 210, 243, and 248 were replaced by asparagines and the glutamates 224 and 252 with glutamines. These residues are indicated in boldface type in Figure 3. The bacteria carrying the mutant genes were cultured on a small scale and the expressed proteins were analyzed by SDS−PAGE. It appeared that all variants were expressed at levels comparable to the wild-type enzyme (not shown). Activity assays at 40 °C using crude cell lysates showed that these lipases had residual activities of less than 5% in the absence of calcium ions, whereas at a concentration of 10 mM calcium full wild-type activity was found. The results show that these variants are as active as WT-SHL and still bind calcium, and therefore, it is unlikely that the replaced amino acids are involved in calcium binding.

Finally we used yet another approach to identify a second calcium ligand in SHL. In the structure of PGL Asp287 is located at hydrogen-bonding distance of Asn284 (*15*). In the open structure of the lipase from *Pseudomonas cepacia* (a close homologue of PGL), Asn284 makes a hydrogen bond with a water molecule, which is one of the calcium ligands (*29*). Interestingly, an aspartate instead of an asparagine is found at this position in all staphylococcal lipases and in BTL. Because it is conceivable that the presence of two acidic residues at a buried position in the active-site loop requires the presence of a calcium ion so as to avoid deformations in this loop, we replaced Asp354 of SHL with asparagine and lysine. The activity of both purified variants was determined in the absence and presence of calcium ions. From the data presented in Table 1 it is clear that the activity of both variants is virtually calcium-independent, suggesting that Asp354 is indeed involved in calcium binding.

From the data in Table 1, it is also obvious that mutations in positions 354 and 357 resulted in reduced activities at 40 °C compared to WT-SHL. To investigate this phenomenon more closely, we determined the temperature profiles of the variant lipases, both in the presence and in the absence of calcium. The resulting curves for D357E-SHL and D357K-SHL are shown in Figure 5, upper panel. It can be seen that the D357E variant behaves very similarly to the wild-type lipase, and especially at higher temperatures the enzyme needs calcium ions for full activity. It should be noted that the activity of D357E-SHL at room temperature is even slightly higher compared to wild-type activity in the presence of calcium (cf. Figure 5, upper panel, and Figure 1). However, at 40 and 50 °C the activity of D357E-SHL is clearly reduced compared to that of WT-SHL (Table 1; cf. Figure 5, upper panel, and Figure 1). At room temperature the calcium-independent enzymes D357K (Figure 5, upper panel) and also D357N-SHL and D357A-SHL, which have activity profiles like that of the D357K variant (data not shown), have activities like that of WT-SHL in the presence of calcium. However, these three calcium-independent variants are more sensitive than D357E to increases in temperature, resulting in strongly reduced activities at higher temperatures. Nevertheless, the activities of these four variants exceed the activity of wild-type apoenzyme over the whole temperature range.

The curves obtained for the variants D354N and D354K are depicted in Figure 5, lower panel. Compared to the curves of the D357K (or D357N and D357A) variants the curves of the D354N and D354K variants are quite different and do not show an optimum at room temperature. The profile

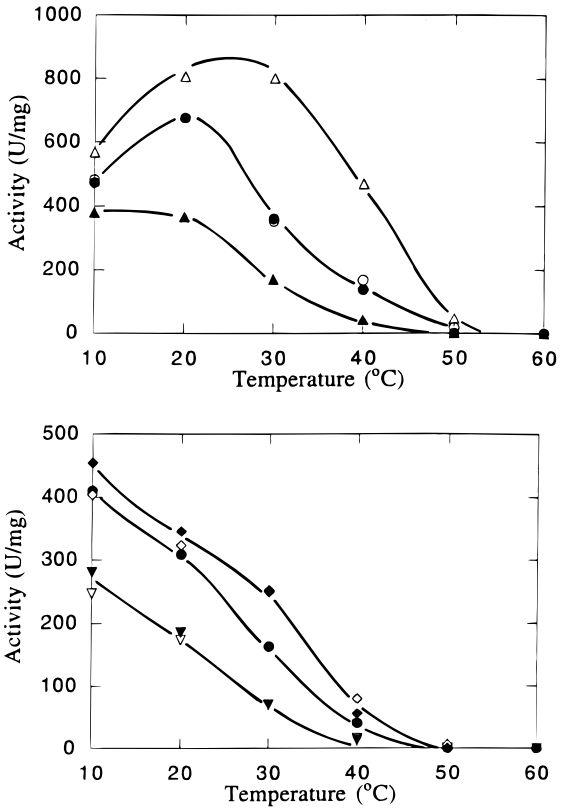

FIGURE 5: Temperature profiles for the activities of WT-SHL and variant SHLs in the presence and absence of calcium. The upper panel shows profiles for D357E-SHL in the presence of either 10 mM $CaCl_2$ (△) or 5 mM EDTA (▲) and for D357K-SHL in the presence of either 10 mM $CaCl_2$ (○) or 5 mM EDTA (●). The calcium-independent profiles of variants D357N and D357A are within experimental error identical to that of D357K-SHL and are not shown for reasons of clarity. The lower panel shows the profiles for WT-SHL in the presence of 5 mM EDTA (●), for D354K-SHL in the presence of either 10 mM $CaCl_2$ (▽) or 5 mM EDTA (▼), and for D354N−SHL in the presence of either 10 mM $CaCl_2$ (◇) or 5 mM EDTA (◆). The composition of the substrate solutions was as described in the legend of Figure 1.

of the variants D354N and D354K are similar to WT-SHL in the absence of calcium ions, suggesting that the calcium binding site indeed has been removed, resulting in a destabilized structure. The observation that mutations at position 354 give rise to apoenzyme activity while mutations at position 357 result in activities higher than apoenzyme activity will be discussed in the next section.

DISCUSSION

The activity of several lipolytic enzymes is regulated by calcium ions. For example, in secretory phospholipases A_2, calcium ions are involved in the binding of the substrate and in the polarization of the scissile ester bond and it has been concluded that their primary role is a catalytic one (*30*, *31*). Several lipases have been reported to be calcium-dependent too, but here the role of calcium is less well-defined. As early as 1968 the first report appeared on the activation of porcine pancreatic lipase by calcium (*32*). When bile salts are present this enzyme requires calcium ions for activity, and the author concluded that calcium is bound by the enzyme. That very year a paper appeared from the same group where the influence of calcium ions on the titration efficiency of liberated fatty acids was described in detail (*33*). In this paper the authors showed that calcium ions partially prevent the inhibitory effect of long-chain fatty acids. The impact of this paper has been that it nearly has become a dogma (ref *34* and references therein) to ascribe the activating effect of calcium ions to their influence on the product rather than to effects at the protein level, and most lipases have been assayed ever since in the presence of added calcium ions even with short-chain substrates.

Little or no attention has been paid to the presence of calcium ions in the structure of pancreatic lipases. However, one observation by Gubenator et al. (*35*) of a calcium ion in the structure of human pancreatic lipase, at a site remote from the active site, seems to support the original notion by Benzonana (*32*) that porcine pancreatic lipase contains a high-affinity calcium binding site. This additional role for calcium ions in pancreatic lipase is supported by a recent publication by Wickham et al. (*36*). The authors concluded that calcium is present in a catalytically active enzyme−mixed micelle−calcium ion complex. For bacterial lipases the localization of calcium ions has been well-established for lipases from the *Pseudomonas* family only. In PGL a calcium ion has been localized in the X-ray structure, from which it was concluded that the presence of this ion stabilizes the structure of PGL (*15*). This finding explained the observation that calcium ions protect PGL against the action of proteases (*37*). On the basis of several observations it can be concluded that SHL also binds calcium ions to stabilize its structure. First, calcium protects the protein against urea-induced unfolding (*18*). Second, the activity is high in the presence of calcium, while it remains at a level of 2−5% in the absence of calcium (*16*, *26*), and third, this calcium dependence is influenced by the reaction temperature (this study). Using ITC we have now been able to measure this calcium binding with a biophysical technique. Our results show that SHL has one high-affinity calcium binding site. For the homologous thermostable lipase BTL, preincubation with EDTA does not affect the activity (*27*), but the 3-fold reduced activity with EDTA instead of calcium in the assay and the conserved acidic residues, which we observed, suggests that this enzyme also binds calcium ions.

The micromolar affinity of SHL for calcium is indicative of a binding site that involves the concerted action of at least two negatively charged amino acids (*28*). This raised the question whether it is possible to localize residues in SHL involved in this binding. Ideally such residues can be identified on the basis of structural analysis, but no X-ray structure of SHL is available. However, all lipases of known three-dimensional structure belong to the superfamily of α/β-hydrolases and we assumed that the core of SHL can be superimposed on that of lipases of known structure. For this comparison we selected the lipase from *P. glumae*, since it is a homologous bacterial lipase and because in the X-ray structure of PGL a calcium ion has been localized. One of the calcium binding aspartates is located in close proximity to the conserved active-site histidine. Since an aspartate is present at this position in the sequence of all staphylococcal lipases and BTL, we assumed that this residue also binds calcium in the case of these latter enzymes. This was confirmed for SHL by a 30-fold decrease in affinity as a result of the single amino acid substitution Asp357 to Glu. Although the negatively charged glutamate partially takes over the function of the aspartate, the exact position of the

charge appears to be very important for efficient binding of the calcium ion. This result illustrates the high specificity of these calcium−protein interactions. In PGL the second aspartate is located remote from the active site, and the calcium ion probably stabilizes the structure by bridging the active-site region to a second subdomain of the protein, thus maintaining the integrity of the complete enzyme and conferring resistance against proteolysis (*15*). The finding that the two calcium ligands of SHL (Asp354 and Asp357) both are localized in the active-site loop, rather than being present in two different loops, underscores the notion that SHL and PGL have evolved with a high degree of homology but that there are major structural differences in the organization of the subdomains. Furthermore, such a spatial arrangement might explain that calcium ions do not protect SHL against the action of trypsin and chymotrypsin (data not shown).

The presence of stabilizing calcium binding sites is not restricted to lipolytic enzymes. An example is the coagulation protease protein C. For this enzyme it has been shown that replacement of one of the two calcium binding glutamates by a lysine yielded a calcium-independent enzyme (*38*). On the basis of this experiment the authors proposed that this mutation probably resulted in a salt bridge that fixed the enzyme in a conformation identical to the calcium-stabilized conformation. Also in SHL the mutation of both Asp354 and Asp357 resulted in calcium-independent enzymes, but it is unlikely that these calcium-independent activities result from the formation of a salt bridge. The latter conclusion is based on two observations. First, we have constructed the control variants D354N-SHL, D357N-SHL, and D357A-SHL demonstrating calcium-independent activity, and second, we showed that both D354K-SHL and D357K-SHL have reduced thermostabilities. The different stabilities of the D354 variants and the D357 variants might reflect the orientation of the side chains, which is probably internal for Asp354 and more exposed for Asp357.

Our observation that the three calcium-independent variants D357K, D357N, and D357A are as active as WT-SHL at room temperature raises the question why this enzyme has evolved a calcium binding site. The advantage of calcium binding becomes evident at higher temperatures. Under such conditions WT-SHL is strongly stabilized by the binding of calcium, and both the activity and the thermostability of the holoenzyme by far exceed those of the calcium-independent variants. These results confirm our earlier hypothesis that SHL binds calcium to stabilize its structure, which is especially significant at higher reaction temperatures. A strong decrease in thermostability was also observed upon engineering of calcium-independent variants of the protease subtilisin BPN′ by removing the calcium binding site (*39*). Interestingly, for this enzyme it was recently shown that this loss in thermostability can successfully be overcome by using an approach of directed evolution, hereby introducing additional, stabilizing mutations (*40*). By using a similar approach the thermostability of our calcium-independent SHL variants might also be further improved. Alternatively, to construct a thermostable calcium-independent lipase, one could start from the thermostable BTL.

In this study we have concentrated on staphylococcal lipases with the structure of PGL, which contains a high-affinity calcium binding site, as a lead. These sites are present in all other *Pseudomonas* lipases and they can be observed either directly in the X-ray structure or from sequence alignment (*29*, *41*−*43*). The conflicting results that have been published for many lipases with respect to their requirement for calcium as a cofactor have so far mainly been ascribed to an increased titration efficiency of fatty acids. We propose that calcium may be involved in the stabilization at the protein level of many more lipases than recognized so far.

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