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Physical and chemical characterization of the oligomerization state of the *Aeromonas hydrophila* lipase/acyltransferase

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Aeromonas glycerophospholipid:cholesterol acyl transferase undergoes a conformational transition upon activation by treatment with trypsin. Chemical cross-linking and sedimentation velocity analysis showed that the lipase dimerizes due to removal of a region near its C-terminus. The lipase monomer has a sedimentation coefficient $s_{20,w}$ = 2.83 S, whereas the dimer has $s_{20,w}$ = 3.65 ± 0.22 S. Hydrodynamic analysis using these sedimentation values and the masses determined by mass spectrometry indicated that the monomers are aligned side-by-side in the dimer. An important change occurs in the apparent partial specific volume of the molecule upon activation.

Lipase; Acyltransferase; Activation; Dimerization; *Aeromonas*

1. INTRODUCTION

In spite of the widespread attention that lipases have received, there is still considerable uncertainty about how they interact at the lipid–water interface, and few details of their reaction mechanisms are clearly understood. The lipase/acyltransferase secreted by *Aeromonas* sp. has several unusual features [1,2]. Although it hydrolyzes a variety of lipid substrates, it preferentially catalyzes acyltransfer in aqueous systems containing an appropriate acyl acceptor. In this way it is similar to the mammalian plasma enzyme, lecithin:cholesterol acyltransferase (LCAT). Like LCAT, the microbial enzyme has no cation requirement, and in mixtures of cholesterol and phosphatidylcholine, acyl transfer occurs almost entirely from the 2-position of the phospholipid.

Recently we have shown that *Aeromonas* lipase is released in a form which cannot attack lipids in membranes [3]. This appears to be due to the fact that it is unable to penetrate lipid bilayers at pressures above 20 mN/m: biological membranes are thought to have pressures above 30 mN/m. Outside the cell, the protein can be nicked by proteases such as trypsin, leading to the loss of a peptide of about 4 kDa. After this occurs, the enzyme is active at surface pressures up to 40 mN/m. Our preliminary evidence suggested that trypsin treatment leads to dimerization of the protein. Here we identify the region of the molecule removed by the protease, and we further characterize the conformational transition that follows.

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Abbreviations: DMS, dimethylsuberimidate; LCAT, lecithin: cholesterol acyltransferase; T-lipase, trypsin-activated lipase.

2. MATERIALS AND METHODS

2.1. *Protein purification and activation with trypsin*

Glycerophospholipid:cholesterol acyltransferase was obtained from cultures of *Aeromonas salmonicida* CB3 (pJT2). The conditions for the growth of the bacterial strain and the purification of the protein have been described [3]. The purified lipase was activated by treatment with trypsin following the procedure outlined in [3]. Complete conversion to T-lipase was confirmed by SDS-PAGE electrophoresis.

2.2. *Chemical cross-linking*

Chemical cross-linking of the lipase before and after treatment with trypsin were performed with dimethyl suberimidate in the same way as we have described elsewhere for the cross-linking of the channel-forming toxin aerolysin [4].

2.3. *Analytical ultracentrifuge analysis*

Sedimentation equilibrium and sedimentation velocity experiments were carried out in a Model-E Analytical Ultracentrifuge with a computer-interfaced scanner (Ultrascan Interface and Data Analysis Program Version 1.70; Borries Demeler, Computer Research and Application, Missoula, MT). The scanning was performed at 282 nm. Sedimentation equilibrium runs were carried out at 30,000 rpm in an An-F rotor and sedimentation velocity runs were carried out at 44,000 rpm using the same rotor. The temperature was 16–17°C throughout all runs. Sedimentation velocity analysis was carried out according to the method of van Holde and Weischet [5]. The partial specific volumes ϕ_2^* of the proteins were calculated using the data from the sedimentation equilibrium runs and the molecular masses determined by mass spectroscopy (see below), according to:

$$\phi_2^* = \left(1 - \frac{2RT}{M_{ms} \cdot \omega^2} \cdot \frac{d\ln c_2}{dr^2}\right) \frac{1}{\rho} \quad (1)$$

where ϕ_2^* = apparent partial specific volume of the protein, R = gas constant, T = absolute temperature, M_{ms} = molecular mass determined by mass spectroccopy; ω = angular velocity, r = radial distance (in cm), c_2 = protein concentration, and ρ = density of the solution.

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Partial specific volumes were also evaluated from the amino acid composition of the proteins according to Perkins [6]. Conformational analysis was performed as described elsewhere [4,7].

2.4. *Mass spectrometry*

The molecular masses of the lipase and T-lipase were determined by matrix-assisted laser desorption ionization using a Kratos Kompact MALDI III, V2.0.1 (Kratos Analytical) time of flight spectrometer with a cyano-4-hydroxycinnamic acid matrix.

2.5. *Gel electrophoresis*

SDS-polyacrylamide electrophoresis was carried out according to Neville [8].

3. RESULTS

3.1. *Chemical cross-linking of the lipase and T-lipase*

Fig. 1 shows the results obtained when the lipase and T-lipase were treated with DMS and subjected to SDS-polyacrylamide gel electrophoresis. Before cross-linking, both forms of the enzyme migrated as single bands, as we have reported earlier [3]. Treatment of the lipase with the cross-linking agent had no effect on its mobility, however, similar treatment of T-lipase resulted in the appearance of a second band with an apparent molecular weight corresponding to a T-lipase dimer. The relative proportions of the two bands remained the same when cross-linking was performed at lower concentrations of T-lipase, indicating that dimer band was not the result of non-specific associations in solution.

3.2. *Analytical ultracentrifugation*

Sedimentation velocity analysis of the two forms of the protein (Fig. 2) clearly showed that both were monodisperse, and the sedimentation coefficients ($s_{20,w}$ = 2.83 S for the lipase and $s_{20,w}$ = 3.65 ± 0.22 S for T-lipase) supported the conclusion from the cross-linking results that they are in different oligomerization states. In fact, the absence of any lines converging toward $s_{20,w}$ = 2.8 S in the case of T-lipase indicates that there were no monomers of this form of the enzyme in the absence of SDS. Sedimentation equilibrium analysis of the lipase and T-lipase showed linear $d\ln c_2/dr^2$ plots in both instances.

3.3. *Mass spectrometry of the lipase and T-lipase*

The precise molecular masses of the lipase and T-lipase were determined by mass spectrometry (see Fig. 3). The value obtained for the lipase (see also Table I) is in very good agreement with that expected from the amino acid sequence [3]. The difference in mass between the lipase and T-lipase corresponds to a peptide of approx. 3,550 Da. This must mean that trypsin cuts the enzyme at R238 and R274. We had previously established the latter site by N-terminal amino acid sequencing of T-lipase [3].

3.4. *Conformational parameters*

Combining the molecular mass values obtained by mass spectrometry with the sedimentation equilibrium data enabled us to estimate the apparent partial specific volumes of the proteins (see eqn. 1 in section 2). It may be seen in Table I that there is a striking change in ϕ_2^* upon activation. The sedimentation coefficients of the lipase and T-lipase and their corresponding masses were used to determine the conformational parameters of the molecules. They are also shown in Table I.

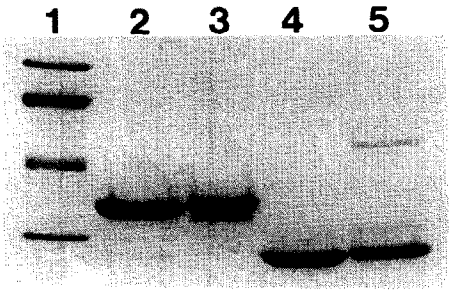

Fig. 1. Chemical cross-linking of the lipase and T-lipase. The proteins (0.5 mg/ml) were treated with 10 mM DMS in 0.1 M triethanolamine, pH 8.5 and separated by SDS-polyacrylamide gel electrophoresis. Lanes 2,3, lipase; lanes 4,5, T-lipase, each before and after cross-linking. Molecular weight markers (97, 66.2, 45, 31 kDa) are in lane 1.

4. DISCUSSION

The sedimentation coefficient of the lipase monomer ($s_{20,w}$ = 2.83 S, Table I is in very good agreement with the value of 2.76 S independently determined previously [3]. As can be seen in Fig. 3A, all the lines converge to a unique s value, indicating that the sample was completely monodisperse. Although the T-lipase dimer also appeared to be monodisperse, upon extrapolation of s_{app} to $t^{-1/2} = 0$, a small amount of scattering corre-

Table I

Conformational parameters of the lipase and T-lipase

Parameter	Lipase	T-Lipase
Molecular mass[a]	35,108 (35,100)	31,649 (×2) [63,000]
ϕ_2^* ($\bar{v}_2$), cm^3 · g^{-1}[b]	0.754 (0.734)	0.780 (0.732)
$s_{20,w}$, S	2.83	3.65 ± 0.22
f/f_0[c]	1.23	1.24 ± 0.08
R_o, Å[c]	21.9	26.9
R_s, Å[c]	26.9	33.3
a/b[b,c]	4.7	4.9 ± 1.2
a, Å[c,d]	61.4	77.1 ± 12.6
b, Å[c,d]	13.1	15.9 ± 1.4
R_G, Å[c,d]	39.3	49.3 ± 7.8

[a] Molecular mass determined by mass spectrometry. Values in parentheses were established from the sequence.

[b] Apparent partial specific volume (ϕ_2^*) determined as described in section 2. Partial specific volume ($\bar{v}_2$) values in parentheses were estimated from the amino acid analysis composition of the proteins as described in [4].

[c] The experimentally determined ϕ_2^* value was used in all these calculations.

[d] Calculated assuming a prolate ellipsoid shape as in [4].

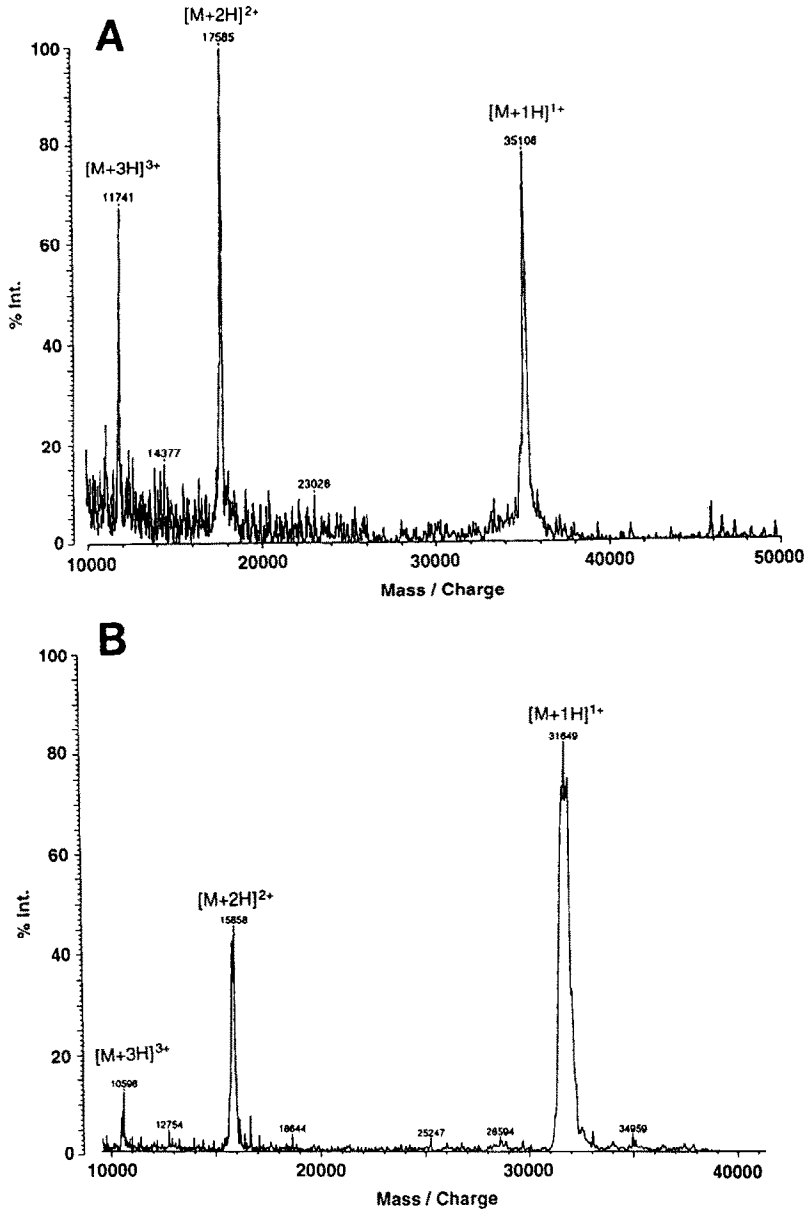

Fig. 2. Matrix-assisted laser desorption ionization spectra of (A) lipase and (B) T-lipase.

sponding to about ± 0.2 S (such as that shown in Fig. 3B) was consistently observed at the point of convergence. The breadth of this scattering, and the complete absence of any lines converging toward the monomer s_{app} value (~ 2.8 S) indicate that it cannot be accounted for by the presence of a mixed population of T-lipase monomer and dimer forms. It most likely reflects the presence of some conformational microheterogeneity of the dimer such as that illustrated in the insert of Fig. 3B.

The apparent partial specific volumes of both the lipase and T-lipase are different from those determined from their amino acid sequences (see Table I) and in addition, there is an important change accompanying oligomerization. In a three-component system, ϕ_2^* can be related to the preferential hydration parameter ξ_1 of a protein by the following equation [9]:

$$1 - \phi_2^* \rho = (1 + \xi_1) - \rho(\bar{v}_2 + \xi_1 \bar{v}_1) \quad (2)$$

with

$$\xi_1 = B_1 - \frac{B_3 - E_3}{w_3} \quad (3)$$

where $\bar{v}_1$ = partial specific volume of water, $\bar{v}_2$ = partial specific volume of the protein, ρ = density of the solution, B_1 = water binding (hydration), B_3 = salt binding, E_3 = salt excluded by Donnan effects, and w_3 = molality of the salt in the buffer.

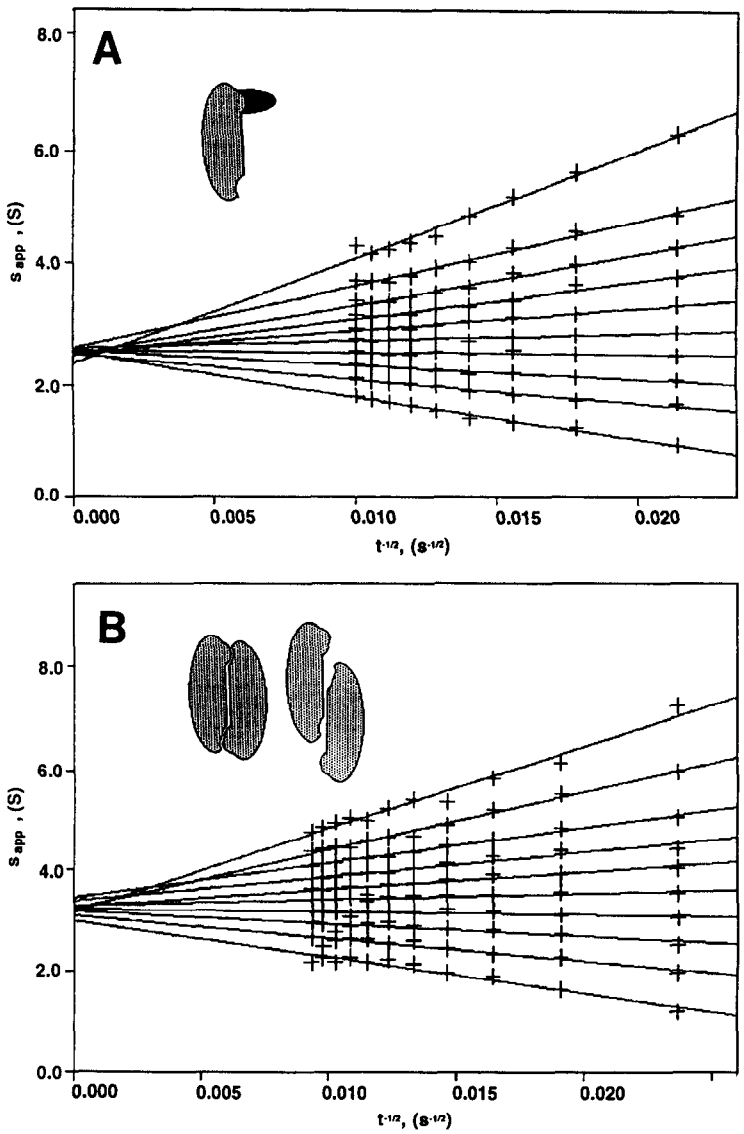

Fig. 3. Sedimentation velocity analysis of (A) lipase and (B) T-lipase. The buffer was 200 mM NaCl, 20 mM Tris-HCl (pH 7.4). The absorbance of the protein at 280 was 0.8 in A and 0.6 in B. In this kind of analysis the number of lines converging toward a common s_{app} value is proportional to the fraction of sample represented [5]. Also shown is a cartoon representation of the lipase before (A), and after (B) activation by trypsin. The dark shaded region represents the fragment of the protein removed by trypsin. After activation the protein dimerizes (B). The conformational microheterogeneity exhibited by some of the dimers is also illustrated in the cartoon.

It may be seen that the change in ϕ_2^* upon dimerization of T-lipase must reflect either changes in hydration and/ or salt binding as a result of the association of monomers. Since the values of ϕ_2^* clearly must be more representative of the properties of the proteins than the theoretical $\bar{v}_2$ values estimated from the amino acid composition, they were used in the conformational calculations shown in Table I.

The results presented in Table I show that the asymmetry of the dimer complex is very similar to that of the monomer, suggesting that the dimerization phenomenon takes place by side-by-side interaction of the monomers along their major axes, as is schematically shown in the insert of Fig. 3B. Why the changes in the conformation and oligomerization state of the *Aeromonas* lipase upon trypsin treatment have such a striking effect on the enzyme's ability to penetrate lipid bilayers is not clear. Perhaps there is a change in surface charge so that the dimer can more easily bind at the lipid–water interface. Whether dimerization serves a similar purpose in other lipases, such as lipoprotein lipase [10], and pancreatic cholesterol esterase [11], also remains to be established.

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