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Premicellar complexes of sphingomyelinase mediate enzyme exchange for the stationary phase turnover

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Abstract

During the steady state reaction progress in the scooting mode with highly processive turnover, *Bacillus cereus* sphingomyelinase (SMase) remains tightly bound to sphingomyelin (SM) vesicles (Yu et al., Biochim. Biophys. Acta 1583, 121–131, 2002). In this paper, we analyze the kinetics of SMase-catalyzed hydrolysis of SM dispersed in diheptanoylphosphatidyl-choline (DC_7PC) micelles. Results show that the resulting decrease in the turnover processivity induces the stationary phase in the reaction progress. The exchange of the bound enzyme (E*) between the vesicle during such reaction progress is mediated via the premicellar complexes ($E_i^{\#}$) of SMase with DC_7PC. Biophysical studies indicate that in $E_i^{\#}$ monodisperse DC_7PC is bound to the interface binding surface (i-face) of SMase that is also involved in its binding to micelles or vesicles. In the presence of magnesium, required for the catalytic turnover, three different complexes of SMase with monodisperse DC_7PC ($E_i^{\#}$ with $i=1, 2, 3$) are sequentially formed with Hill coefficients of 3, 4 and 8, respectively. As a result, during the stationary phase reaction progress, the initial rate is linear for an extended period and all the substrate in the reaction mixture is hydrolyzed at the end of the reaction progress. At low mole fraction (X) of total added SM, exchange is rapid and the processive turnover is limited by the steps of the interfacial turnover cycle without becoming microscopically limited by local substrate depletion or enzyme exchange. At high X, less DC_7PC will be monodisperse, $E_i^{\#}$ does not form and the turnover becomes limited by slow enzyme exchange. Transferred NOESY enhancement results show that monomeric DC_7PC in solution is in a rapid exchange with that bound to $E_i^{\#}$ at a rate comparable to that in micelles. Significance of the exchange and equilibrium properties of the $E_i^{\#}$ complexes for the interpretation of the stationary phase reaction progress is discussed.

Keywords: Sphingomyelinase; *Bacillus cereus*; Interfacial catalytic turnover; Pseudo-scooting mode; Premicellar complex; Transferred NOESY

1. Introduction

SMase[1] (sphingomyelinase from *Bacillus cereus*) binds with high affinity to sphingomyelin (SM) vesicles, and the enzyme remains tightly bound to the vesicle interface during the highly processive interfacial catalytic turnover in the scooting mode [1]. Thus, during the steady-state in the scooting mode, the bound enzyme does not leave the interface. While the phosphocholine leaves the interface, the ceramide product remains in the target vesicle. At the end of the reaction progress, only the substrate on the external surface of the SMase-containing SM vesicles is hydrolyzed. Substrate present in the inner layer of the enzyme-containing vesicles, as well as in the excess vesicles to which the enzyme is not initially bound, is not hydrolyzed. In analogy with the behavior of secreted phospholipase A_2 such features of the reaction progress in the highly processive scooting mode show that the interface binding step is distinct from the catalytic turnover events [2–5], and that the catalytic site is different than the i-face (the interface binding surface) of the enzyme [6].

Abbreviations: DC_7PC, Diheptanoylphosphatidylcholine; HDNS, N-dansyl-hexadecyl-1-phosphoethanolamine; i-face, the interface binding surface of an interfacial enzyme; ITC, isothermal calorimetry; RET, fluorescence resonance energy transfer; SM, sphingomyelin; SMase, sphingomyelinase from *Bacillus cereus*; TMA-DPH, trimethylammonium-diphenylhexatriene

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Operationally, the i-face of an interfacial enzyme is designed to bind organized interfaces of amphiphiles. Therefore, it is not surprising that the i-face should also have a tendency to bind and organize monodisperse amphiphiles to form premicellar complexes as shown for phosphatidylinositol specific phospholipase C [7] and phospholipase A_2 [8,9]. Fig. 1 is a paradigm for this concept of premicellar $E_i^{\#}$ complex formation of interfacial enzymes with monodisperse amphiphiles (A), and also for understanding the relationship between the $E_i^{\#}$ complexes and the E* form at the preformed amphiphile interfaces (A*) such as micelles and bilayer vesicles. When A is SM, the concentration of monodisperse A will be very low not exceeding CMC ($<10^{-12}$ M). Under these conditions, both A and E will be more likely to go to the interface rather than forming $E_i^{\#}$; thus only E and E* will coexist in this case. However, if one component of a (mixed-)micellar dispersion is in significant monodisperse concentration, then E, $E_i^{\#}$ and E* can all coexist. On the other hand only E and $E_i^{\#}$ can coexist below the CMC with only the A form present.

In this paper, we analyze functional consequences of the coexistence of E, $E_i^{\#}$ and E* (Fig. 1) forms of SMase in the presence of DC_7PC. Not only is DC_7PC bound to $E_i^{\#}$ in rapid exchange with the monodisperse amphiphile in the aqueous phase, but the $E_i^{\#}$ complex mediates a rapid exchange of SMase between the coexisting SM-containing interfaces which gives rise to the stationary phase in the reaction progress. Although the interfacial binding of the enzyme remains strong, the presence of monodisperse amphiphile and the concomitant formation of $E_i^{\#}$ complex allow rapid exchange of enzyme between micelles. As modeled in Appendix, the kinetic consequence is that the magnesium-dependent catalytic turnover by SMase occurs over an extended period of time without local depletion of the substrate on the enzyme-containing interface. Results are quantitatively interpreted with the assumption of ideal mixing of SM with the partitioned DC_7PC. Our analysis shows that not only do the $E_i^{\#}$ complexes mediate rapid exchange of the enzyme between the coexisting substrate-containing interface, but at >0.8 mole fraction DC_7PC is also an ideal surface diluent for the SM interface. Our results show that the cooperative binding of monodisperse DC_7PC molecules to the i-face of the enzyme sequentially forms the three premicellar complexes ($E_i^{\#}$). Since magnesium is required for the observed SMase-catalyzed hydrolysis [1,10,11], these results suggest an obligatory role for the cation in the binding of the enzyme to the interface. The dissociation constants for the bound DC_7PC and the transferred NOESY results provide insights into the rapid exchange of A from $E_i^{\#}$ and micelles, presumably as multi-step fusion–fission kinetic process facilitated by step-wise removal of single amphiphile molecules.

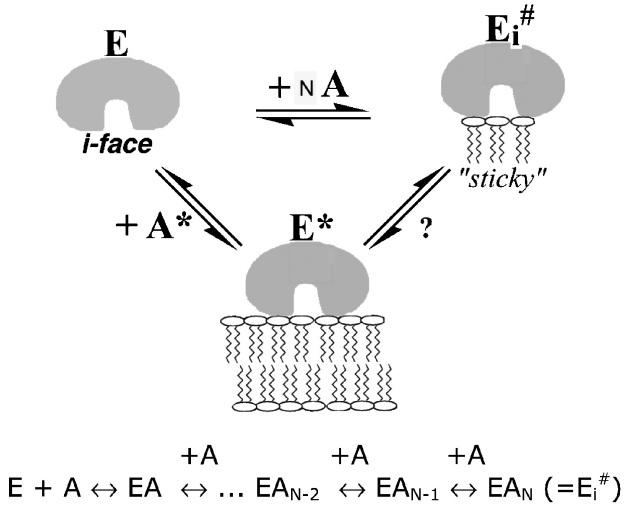

Fig. 1. A cartoon to conceptualize the cooperative binding of N monodisperse A (DC_7PC) or of aggregated A* amphiphiles to the i-face of SMase in the aqueous phase (E) to form $E_i^{\#}$ or E* complexes without the occupancy of the active site. Based on the evidence in this paper, $i=1, 2, 3$ for the three sequentially formed premicellar $E_i^{\#}$ complexes of SMase as shown by the fluorescence changes from the protein. On the other hand, as also shown in this paper, the amphiphile exchange rate monitored as NOESY signal is related to the association and dissociation of individual amphiphiles, i.e., $E_i^{\#}+A \leftrightarrow E_i^{\#}$ ($i=1, 2, 3$). In analogy to the micellization [4], this is one of the steps in the sequential equilibria as expanded in the lower part of the figure. Similar exchangeable discrete premicellar $E_i^{\#}$ that may represent the sub-states between E and E* are also formed with other interfacial enzymes [7–9] suggest that only certain discrete complexes.

2. Material and methods

2.1. Reagents

DC_7PC was from Avanti. All other reagents were analytical grade. A preparation of SMase, expressed from the cloned SMase gene from *Bacillus cereus*, was purchased from Higata Shoyu Co. (Japan). It was further purified as described earlier [1], where we used the OD_{280} of 23 for 1% SMase solution. Based on the W, Y and F content OD_{280} is 16, and therefore based on this value, the turnover rates in this report are apparently 30% lower.

Unless mentioned otherwise, all measurements were carried out in 20 mM Tris buffer and 10 mM NaCl at pH 8 and 24 °C with indicated amount of added divalent cation. Stock solution of Tris and NaCl were filtered through a bed of Chelex to remove multivalent cations. The concentration of magnesium if present was kept at 3 mM that is at least 10 times above the apparent K_d for magnesium for catalysis as well as for the binding of the DC_7PC monomers. It is also sufficient to compete out trace amount of potent inhibitory cations in buffer. Control buffers in the absence of the divalent cation included 1 mM EDTA and 1 mM EGTA.

2.2. Kinetic measurements

Reaction progress for the hydrolysis of sonicated or extruded SM vesicles was monitored by pH-stat titration as