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Phospholipase A_2 Engineering. 4. Can the Active-Site Aspartate-99 Function Alone?[1]

Cynthia M. Dupureur, Tiliang Deng, Jae-Gyu Kwak, Joseph P. Noel, and Ming-Daw Tsai*

Department of Chemistry and
Ohio State Biochemistry Program
The Ohio State University, Columbus, Ohio 43210

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The Asp···His···Ser "catalytic triad" is the key catalytic vehicle for serine proteases.[3] Recently this class of enzymes has been extended to include lipases.[4] While the catalytic roles of His and Ser have been relatively well established,[3] that of Asp has been a subject of debate. At least three possible roles have been suggested for the Asp: (a) orienting the conformation of His;[5] (b) stabilizing the appropriate tautomer of His;[5] and (c) neutralizing the positive charge of His during the reaction.[6] Strong evidence for role b has been provided in a recent study which

(1) For paper 3 in this series, see ref 2. This work was supported by Research Grant GM41788 from the NIH. J.P.N. was the recipient of a Monsanto Biotechnology Fellowship. We thank K. J. Hamilton for purification of Y73S and Y73A and J. K. Myers for purification of Y52F/Y73F. Abbreviations: Y, F, V, S, A, D, and N are one-letter designations of Tyr, Phe, Val, Ser, Ala, Asp, and Asn, respectively.

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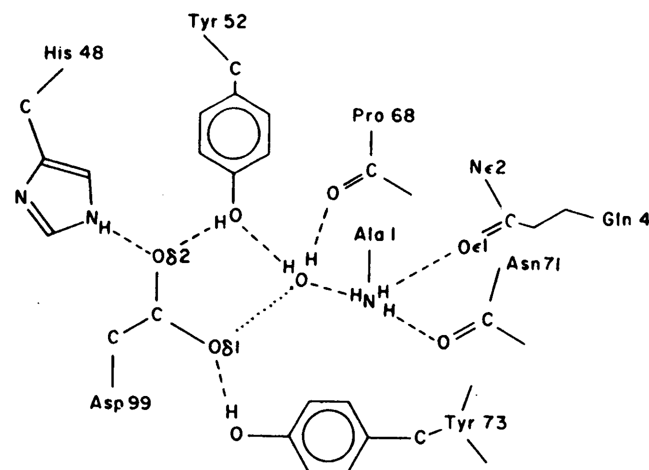

Figure 1. Schematic drawing of the hydrogen-bonding network observed in the 1.7-Å crystal structure of bovine pancreatic PLA2,[12b] as seen previously in ref 12a.

showed that substitution of the Asp-102 of trypsin by Asn caused His-57 to exist in the incorrect tautomer[7] and resulted in a 10^4-fold decrease in k_{cat}/K_m.[8] Some initial evidence for role c has also been presented in a recent meeting.[9] Role a could be inseparable from roles b and c since the latter two may require a proper conformation of His.

Less attention has been given to the observation that, in serine proteases, the carboxylate of the Asp in the catalytic triad is H-bonded to a few other residues. In trypsin, for example, the carboxylate of Asp-102 accepts H bonds from Ser-214 and the backbone amide protons of residues 56 and 57.[10] It is an interesting and important question as to whether these H bonds are required to stabilize the Asp in a proper conformation or the carboxylate of the Asp can perform its catalytic functions without further assistance from other groups.

We examined a similar situation in phospholipase A_2 (PLA2) which uses Asp-99···His-48 as a "catalytic diad", with the role of Ser substituted by water.[11] As shown in Figure 1, the carboxylate oxygens of Asp-99 are H-bonded to Tyr-52 and Tyr-73, which in turn are part of an extensive H-bonding network.[12] The residues His-48, Asp-99, Tyr-52, and Tyr-73, which are invariant among all groups I and II PLA2s (which include all known sequences except bee venom PLA2)[13] and occupy virtually identical positions in all crystal structures,[14] have been collectively termed the "catalytic network" by Sigler.[15] This catalytic network also exists in the crystal structure of a PLA2–inhibitor complex.[16] As depicted in Figure 1, the catalytic network is connected via a water molecule to Ala-1 and Gln-4, which have been suggested to be involved in the "interfacial recognition site" by de Haas.[17]

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Table I. Summary of Kinetic Data[a]

enzyme	DC_8PC micelles: k_{cat}, s^{-1}	K_m, mM	k_{cat}/K_m, M^{-1} s^{-1}	DC_6PC monomers: sp act., μmol^{-1} min^{-1} mg^{-1}
WT	675	1.4	4.8×10^5	5.3
Y52F	265	1.4	1.9×10^5	4.0
Y73F	580	2.0	2.9×10^5	2.1
Y52F/Y73F	205	2.8	0.7×10^5	0.6
Y52V	1.0	0.7	1.4×10^3	0.3
Y73S	1.7	0.9	1.9×10^3	0.4
Y73A	4.0	1.8	2.2×10^3	0.3
D99N	0.6	1.4	0.4×10^3	

[a] Bovine pancreatic PLA2 was isolated from an *E. coli* expression host BL21(DE3)plysS carrying a plasmid pTO-propla2[19] which contained a synthetic gene coding for bovine proPLA2.[18] The procedures for construction and purification of mutants will be described in detail in the future. The assays for both micellar and monomeric substrates were performed at pH 8.0 (1 mM sodium borate, 25 mM $CaCl_2$, 100 mM NaCl) and 45 °C using a pH stat method. The specific activity of monomers was measured by using 5 mM DC_6PC.

Blocking the amino terminus, modifying Gln-4, or changing their immediate environments have been shown to result in total loss of activity toward micellar but not monomeric substrates.[17]

We used site-directed mutagenesis to probe the roles of Tyr-52 and Tyr-73, particularly in relation to the function of Asp-99. Our system is bovine pancreatic PLA2 overproduced in *Escherichia coli*.[18,19] Table I lists the kinetic data for wild-type (WT) PLA2 and mutated enzymes Y52F, Y73F, Y52F/Y73F (double mutation), Y52V, Y73S, Y73A, and D99N.[1] For the micellar substrate DC_8PC (1,2-dioctanoyl-*sn*-glycero-3-phosphocholine), both k_{cat} and K_m have been obtained, whereas for the monomeric substrate DC_6PC, only specific activities were determined because the activities of the mutated enzymes toward the monomeric substrate approach the limit of detection of our assay system. Surprisingly, the kinetic constants for the mutated enzymes Y52F, Y73F, and Y52F/Y73F are perturbed by <10-fold relative to those of WT for both micellar and monomeric substrates. This suggests that Asp-99 is able to perform its catalytic function without being H-bonded to Tyr-52 and Tyr-73. It is possible that other groups or water molecules could move in to H bond to the carboxylate of Asp-99. However, the fact that both Tyr-52 and Tyr-73 can be changed to Phe suggests that such H bonds are most likely unimportant catalytically.

However, the aromaticity of both Tyr residues appears to be required; mutations at positions 52 and 73 involving the loss of aromatic side chain(s) (Y52V, Y73S, and Y73A) resulted in decreases in the catalytic activity by factors of 200–350 for micellar substrates and 10–20 for monomeric substrates. Such changes are likely to be due to structural perturbations since Tyr-52 and Tyr-73 are involved in perpendicular aromatic–aromatic interactions with Tyr-69 and Tyr-75, respectively; such interactions may serve to stabilize the tertiary structure of proteins.[20] Alternatively, the aromatic rings could be involved in carrying water molecules via H bonding.[21] Structural characterization of these mutated enzymes will be required to test these interpretations.

The mutated enzyme D99N showed a 10^3-fold decrease in k_{cat}/K_m, which is comparable to the result reported for the analogous mutation (D102N) in trypsin.[8] Future structural characterization is needed to determine whether this is due to the incorrect tautomeric form of His-48, as in the D102N mutated enzyme of trypsin.[7]

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The above results suggest the following conclusions: (a) The carboxylate of Asp-99 is able to carry out its function without forming H bonds to Tyr-52 and Tyr-73. (b) The phenolic hydroxyl of neither Tyr-52 nor Tyr-73 is catalytically essential even though they are absolutely conserved in groups I and II PLA2 sequences. (c) The aromatic rings of both residues are required, possibly for structural reasons. (d) If the H-bonding network shown in Figure 1 is really important in interfacial catalysis,[13a,17d] it should not involve Tyr-52 or Tyr-73.