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Phospholipase A_2 Engineering. 6. Single Amino Acid Substitutions of Active Site Residues Convert the Rigid Enzyme to Highly Flexible Conformational States[1]

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The effect of single amino acid substitutions on the structure of a protein has been a subject of great interest in structural studies (as in the Ile-6-Met T4 lysozyme whose crystal structure displayed multiple conformations[2]) or of great concern in functional studies (as in the Glu-43-Asp Staphylococcal nuclease, which displayed nonlocal conformational perturbations on the basis of NMR[3] and X-ray[4] analysis). We are less concerned with conformational perturbations with mutants of pancreatic phospholipase A_2 (PLA2, overproduced in *Escherichia coli*)[5] since it is a rigid enzyme consisting of seven disulfide links in a small mass (14 000) and is chemically and thermally stable.[6] However, we present an unprecedented finding: site-directed substitution of two active site residues, Asp-99 to Asn (D99N) and Tyr-73 to Ser (Y73S), each resulting in highly flexible conformations characteristic of (though not identical to) the "molten globule", a conformational state with great flexibility, while still preserving secondary structures.[7]

The partial 1D proton NMR spectra in Figure 1 reveal two important features: (i) The chemical shift dispersion is less extensive for the mutant PLA2s. The chemical shifts are also very different between these spectra. (ii) Most of the nonexchangeable NH protons (which persisted through extensive deuterium exchange and heating to 60 °C) in the 7.8–9.5 ppm region of the wild-type (WT) spectrum have disappeared in Y73S and broadened in D99N. These features, particularly for Y73S, are similar to those observed for the molten globule of α-lactalbumin.[7a]

The differences are even more dramatic in 2D COSY and NOESY spectra. As shown by the partial NOESY spectra in

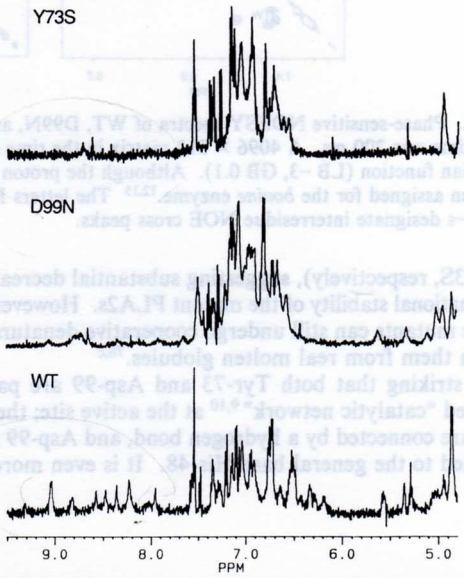

Figure 1. Proton NMR spectra (500 MHz) of WT PLA2, D99N, and Y73S in D_2O. Sample conditions: 1.0 mM enzyme, 50 mM $CaCl_2$, and 300 mM NaCl, pH* 4.0–4.1 at 37 (WT and D99N) and 47 °C (Y73S). The FID was processed with Gaussian multiplication (LB −5, GB 0.1).

Figure 2, only one of the aromatic spin systems, Fc (Phe-94), can be clearly identified in the mutants. The remaining aromatic spins have either shifted significantly or were undetectable. Such results have been corroborated by COSY experiments. For both D99N and Y73S, there are no obvious interresidue NOE cross peaks in the aromatic–aromatic region and very few in the aromatic–aliphatic region. Other regions display similar properties. Lowering the temperature of Y73S by 20 deg improved the spectra only slightly. Other mutants at the same positions, D99A and Y73A, showed similar proton NMR properties.

There is no substantial difference between the circular dichroism (CD) spectra (200–250 nm) of WT and the mutant enzymes, which suggests preservation of secondary structure as is characteristic of molten globules.[7] When CD was used to monitor denaturation induced by guanidine–HCl, the free energy of denaturation,[8] $\Delta G_d^{H_2O}$, of both D99N and Y73S decreased by ca. 2-fold relative to WT (9.5, 4.7, and 4.6 kcal/mol for WT, D99N,

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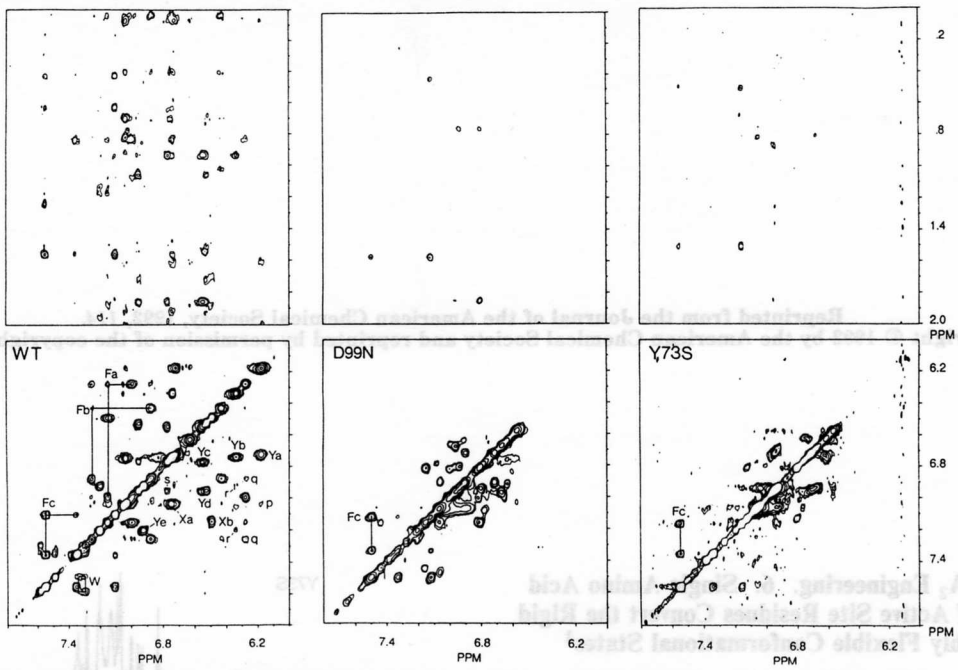

Figure 2. Phase-sensitive NOESY spectra of WT, D99N, and Y73S in D_2O at 500 MHz. The sample conditions are the same as in Figure 1. The mixing time was 200 ms. A 4096 × 512 matrix in the time domain was recorded and zero-filled to a 4096 × 2048 matrix prior to multiplication by a Gaussian function (LB −3, GB 0.1). Although the proton resonances of *porcine* PLA2 have been assigned completely,[14] only limited spin systems have been assigned for the *bovine* enzyme.[12,15] The letters F, Y, and W represent Phe, Tyr, and Trp, respectively, while X is uncertain. The small letters p–s designate interresidue NOE cross peaks.

and Y73S, respectively), suggesting substantial decreases in the conformational stability of the mutant PLA2s. However, the fact that the mutants can still undergo cooperative denaturation distinguish them from real molten globules.[7b,c]

It is striking that both Tyr-73 and Asp-99 are part of the conserved "catalytic network"[9,10] at the active site; the two side chains are connected by a hydrogen bond, and Asp-99 is further H-bonded to the general base His-48. It is even more striking that the highly flexible mutants are still functional, though with activities diminished 10–100-fold.[11,12] Although the latter is expected for active site mutants, our results only allow us to conclude that the two residues play critical structural roles; their functional roles remain to be established.

One should not be led to think that the structure of PLA2 is "fragile". None of the site-specific mutations at seven other conserved positions (Tyr-52, Lys-53, Lys-56, Phe-22, Phe-106, Cys-51, and Cys-98) resulted in large structural perturbations. The Y52V (Tyr-52 is also H-bonded to Asp-99) PLA2 showed activity similar to Y73S, but strikingly similar NOESY spectra relative to the WT enzyme.[12] The double mutant C51A/C98A, in which a disulfide bond has been deleted, exhibited normal activity and only a modest decrease in $\Delta G_d^{H_2O}$ (7.2 kcal/mol). Site-specific substitutions of Phe-22 (to Ile and Ala) and Phe-106 (to Ile, Ala, and Tyr), which were involved in three-way aromatic–aromatic interactions along with Phe-5 in the crystal structure,[9,13] resulted in only small and localized changes in NOESY spectra.

The disappearance of NOEs could be caused by either decreased τ_c (such that $\omega_o\tau_c \approx 1$) or increased average internuclear distances. Other approaches are being undertaken to differentiate these possibilities, to uncover structural basis for the observed effects, and to determine if the conformations are induced back upon binding to micelles.

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