

described here is complementary and can be used when the appropriate fluoro compound is not easily available or when a halogenated nitrophenol is the desired product, in which case the Winkel method may give a mixture of products.

The mechanism of this reaction is presumably attack of hydroxide upon the phenolate anion to form the Meisenheimer complex **1** (Scheme I). Not all resonance structures for the phenolate anion are shown, but for this reaction to proceed the most important contributors are those with the negative charge withdrawn to the para carbon and into the nitro group. The importance of the electron-withdrawing nitro group is demonstrated by the failure of *p*-hydroxyacetophenone to undergo this exchange. Although the *p*-acetyl group lowers the pK of phenol from 10.0 to 8.0 (vs 7.2 for *p*-nitro), it is insufficiently electron-withdrawing to promote sufficient electrophilicity at C-1 for the exchange reaction to occur.

The slower reactivity of *o*-nitrophenol relative to the para isomer cannot be due to steric interference from the vicinal nitro group, as 2,4-dinitrophenol exchanges twice as fast as *p*-nitrophenol. Instead the slower rate presumably reflects the fact that the nitro group at C-2 keeps the charge in the phenolate anion closer to C-1 relative to the para isomer, thereby reducing the electrophilicity at C-1. A synthetically useful rate for this exchange can be attained by increasing the hydroxide concentration (see Table I).

The possibility that these exchanges take place at the nitro groups instead of the phenolic oxygen is not feasible mechanistically, but in any case the mass spectra give direct evidence for phenolic oxygen exchange. The fragment resulting from loss of the nitro group from *p*-nitrophenol, $i/e = M - 46$, shows the same fraction of enrichment with ^{18}O as the molecular ion. In addition, all of the exchange reactions in Table I cease after a single oxygen atom has exchanged.

Acknowledgment. This research was supported by a grant from the National Institutes of Health (GM 18938) to W. W. Cleland. This study made use of the mass spectrometry facility of the Chemistry Department of the University of Wisconsin—Madison.

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Phospholipase A₂ Engineering. 6. Single Amino Acid Substitutions of Active Site Residues Convert the Rigid Enzyme to Highly Flexible Conformational States¹

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Received December 17, 1991

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 conformations²) or of great concern in functional studies (as in the Glu-43-Asp Staphylococcal nuclease, which displayed nonlocal conformational perturbations on the basis of NMR³ and X-ray⁴ analysis). We are less concerned with conformational

(1) Supported by Research Grant GM41788 from NIH. The authors wish to thank Xiaoyan Zhang and Yuan Ji for technical assistance. For paper 5 in this series, see ref 13.

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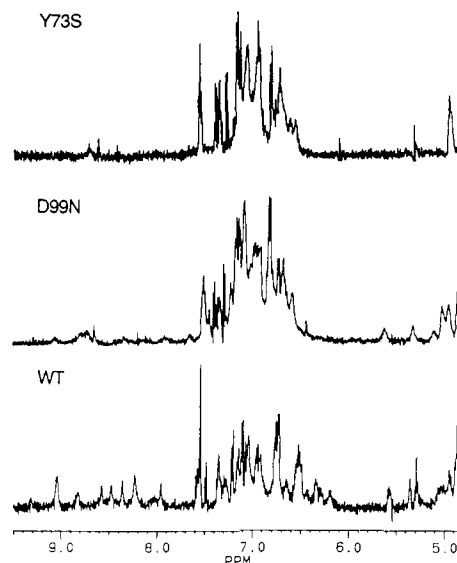


Figure 1. Proton NMR spectra (500 MHz) of WT PLA2, D99N, and Y73S in D₂O. Sample conditions: 1.0 mM enzyme, 50 mM CaCl₂, and 300 mM NaCl, pH* 4.0–4.1 at 37 (WT and D99N) and 47 °C (Y73S). The FID was processed with Gaussian multiplication (LB -5, GB 0.1).

perturbations with mutants of pancreatic phospholipase A₂ (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 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 Δ*G*_d^{H₂O}, 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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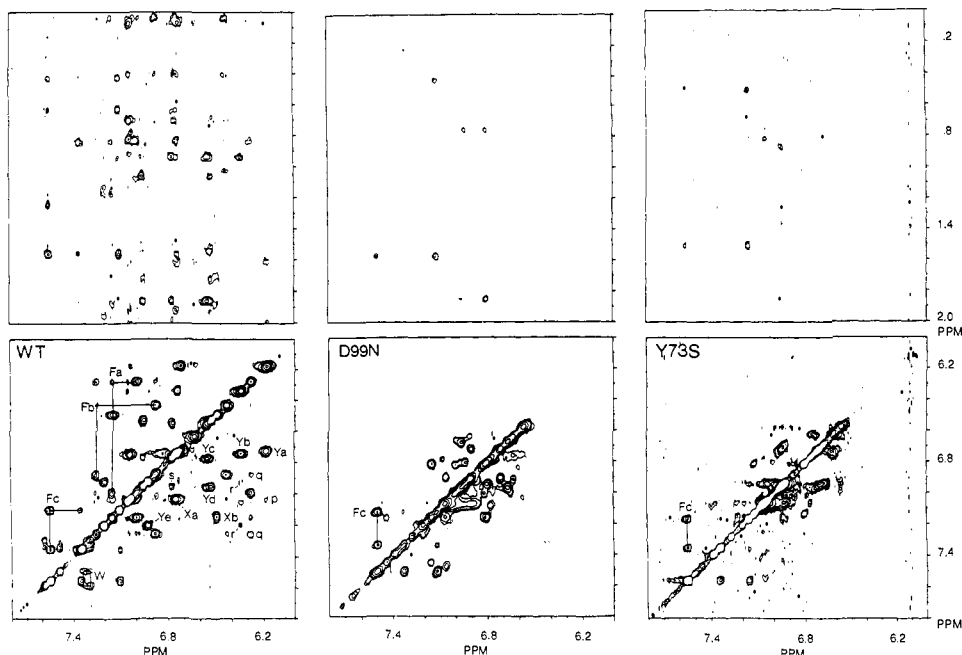


Figure 2. Phase-sensitive NOESY spectra of WT, D99N, and Y73S in D_2O at 500 MHz. The sample conditions are the same as in Figure 1. The mixing time was 200 ms. A 4096×512 matrix in the time domain was recorded and zero-filled to a 4096×2048 matrix prior to multiplication by a Gaussian function (LB -3, 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 $\omega_c \tau_c \approx 1$) 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.

Radical Scavenging in Zeolite Media

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Received December 11, 1991

The model of the solvent cage predicts that the dynamics of dissociating particles in fluid media depends on their probabilities of escape and recombination^{1,2} and that there may be at least three potentially observable stages at which scavenging may occur: (a) primary geminate pairs, (b) secondary geminate pairs, and (c) free radicals.³

Experimental tests^{3,4} have demonstrated scavenging at stages b and c. However, because of the restricted time resolution available within the dynamics of fluid systems, distinctive scavenging at each intermediate stage has not proven possible.⁴ The general concepts of cage effects in liquids have been applied to the dynamic properties of organized media and constrained environments.⁵ In this communication, we report an example where

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