Oxygen-18 Exchange in Nitrophenols: Significance for Labeling and Isotope Effect Experiments

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Nitrophenols labeled with ^{18}O at the phenolic oxygen atom have found use in spectroscopic studies and in the measurement of ^{18}O isotope effects.[1] In addition, the formation of these species in hydrolysis reactions of nitrophenyl esters and phosphates has been used as a mechanistic probe to determine the position of bond cleavage.[2] Nitro-substituted phenols have been reported to undergo only partial exchange of the phenolic oxygen atom with water in the presence of acids or bases, and only then under very harsh conditions.[3] This has led a number of researchers in need of ^{18}O-labeled 2-, 3-, and 4-nitrophenol and 2,4-dinitrophenol to develop a number of synthetic routes to these compounds, some of them rather involved.[1a-e]

The present study shows that, contrary to an earlier report,[3b] phenols bearing a nitro group at the ortho and/or para positions undergo facile exchange of the phenolic oxygen atom with ^{18}O-labeled water in basic solution (Table I). These data signal that caution must be used in mechanistic experiments designed to determine the position of bond cleavage with nitrophenyl substrates. In just such an experiment, the alkaline hydrolysis of the phosphodiester *p*-nitrophenyl 3,3-dimethylbutyl phosphate in ^{18}O-enriched water, 1 N in hydroxide at 100 °C, resulted in the formation of *p*-nitrophenol which was enriched in ^{18}O.[2c] Since *p*-nitrophenol has been reported[3b] to undergo no exchange under far harsher conditions than these, the logical conclusion would be that this reaction occurred via nucleophilic aromatic substitution, resulting from hydroxide attack at the phenolic carbon. Subsequent experiments showed that instead this reaction occurs entirely via hydroxide attack at phosphorus and that the *p*-nitrophenolate thus released had undergone subsequent exchange in solution at a rate faster than its release by hydrolysis.

Similar labeling studies have been used by others to determine the position of bond cleavage in reactions of nitroaryl phosphates and esters. The occurrence of facile exchange at elevated temperatures in basic solution must be taken into consideration in the design and interpretation of these experiments.

Other experiments to which these data have relevance are measurements of kinetic isotope effects on reactions of nitroaryl substrates by isolation of the nitroaryl products for mass spectral analysis.[4] The present results must be taken into account in the design of these experiments as well, as conditions under which exchange occurs must be avoided.

The rate constant for the exchange of *p*-nitrophenol at 100 °C was found to vary in a linear fashion with hydroxide concentration (Figure 1). The exchange rate is 56-fold slower at 50 °C, and a comparison of the rates at 100 and 50 °C gives a value for the activation energy of 19 kcal/mol. If only 1 equiv hydroxide is added to *p*-nitrophenol, no exchange is detected after 72 h, demonstrating that hydroxide and not water is the reactive species. The data for a number of other substituted phenols are also presented in Table I.[5] The first equivalent of hydroxide added in these exchange reactions is consumed in deprotonation of the phenol, and the concentrations of hydroxide shown in Figure 1 and Table I are of net free hydroxide in solution.

This exchange reaction affords a simple means for the preparation of these ^{18}O-labeled compounds.[7] Winkel et al. have reported an efficient synthesis of [^{18}O]-*p*-nitrophenol from *p*-fluoronitrobenzene and [^{18}O]hydroxide.[8] The exchange method

Table I. Exchange Rates of Various Phenols at 100 °C

substrate	[$^-$OH] (M)[a]	k (min^{-1})	$t_{1/2}$ (min)[b]
p-nitrophenol	0.55	9.4×10^{-3}	74
	0.54	1.67×10^{-4} (*c*)	4150
o-nitrophenol	0.55	1.1×10^{-4}	6300
	2.73	5×10^{-4}	1390
m-nitrophenol	0.76	no exchange after 24 h	
2,4-dinitrophenol	0.55	1.9×10^{-2}	36
2-chloro-4-nitrophenol	0.55	9.8×10^{-3} (*d*)	71
phenol	0.62	no exchange	
4-cyanophenol	0.50	(*e*)	
p-hydroxyacetophenone	0.72	no exchange after 24 h	

[a] Net free hydroxide in solution. [b] Half-life for total exchange, calculated from the rate constants in column 3. [c] 50 °C. [d] No displacement of chloride at C-2 by hydroxide could be detected after 5 half-lives. [e] Tested at 100 and 70 °C; hydrolysis of the nitrile group occurs faster than exchange.

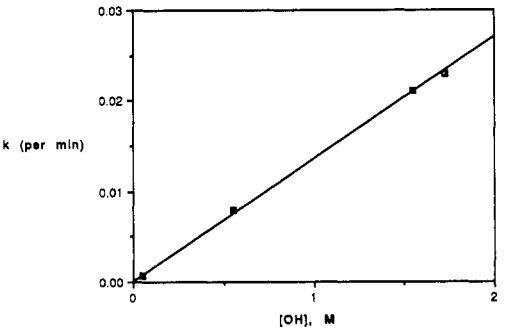
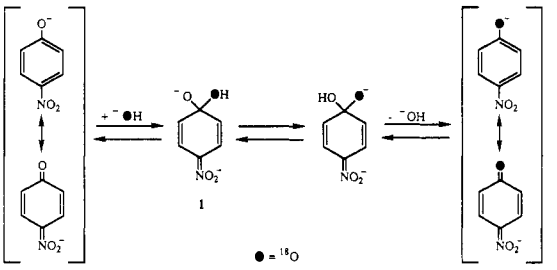


Figure 1. Variation of exchange rate with hydroxide concentration for *p*-nitrophenol at 100 °C.

Scheme I



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(5) Mixtures of the various phenols in 200 μL of ^{18}O-enriched water were heated to 100 °C, and exchange was initiated by adding an aliquot of natural abundance 13 N NaOH solution. Typical reaction mixture concentrations were 0.25–0.50 M in phenolate in 33% [^{18}O]water. At fixed time intervals, 30-μL aliquots were removed and added to 2 mL of cold dilute sulfuric acid solution to stop reaction, and the phenol was isolated by ether extraction and analyzed by mass spectroscopy. A number of measurements of the relative intensities of the molecular ions were made and averaged, and the degree of exchange was calculated from the relative intensities of the molecular ions resulting from the oxygen-16 and oxygen-18 species. Rate constants were obtained by fitting the exchange data to the equation $y = 1 - e^{-kt}$ using the EXPFIT program of Cleland.[6]

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(7) A preparatory-scale reaction was run with *p*-nitrophenol using the same concentrations as for the kinetic runs but on a larger scale. After 10 h, the reaction mixture was allowed to cool, and the enriched water was recovered using a Kugelrohr distillation apparatus at 40 °C under reduced pressure (water aspirator) using a dry ice/acetone bath to cool the receiving flask. The phenolate was taken up in water, acidified, and isolated by ether extraction. The quantitatively recovered labeled product was pure by NMR analysis.

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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 p*K* 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.

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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]

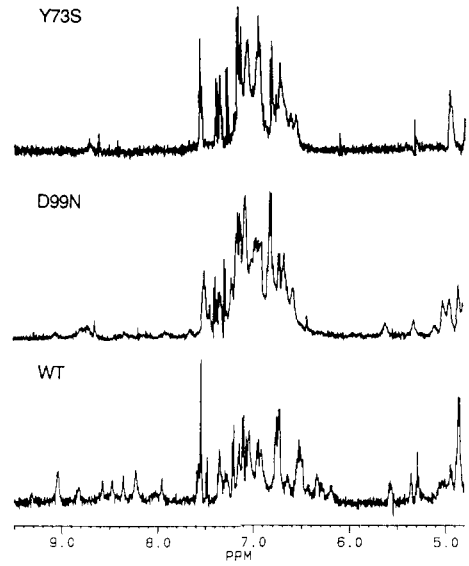

Figure 1. Proton NMR spectra (500 MHz) of WT PLA2, D99N, and Y73S in D_2O. Sample conditions: 1.0 mM enzyme, 50 mM $CaCl_2$, and 300 mM NaCl, pH* 4.0-4.1 at 37 (WT and D99N) and 47 °C (Y73S). The FID was processed with Gaussian multiplication (LB -5, GB 0.1).

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] $\Delta G_d^{H_2O}$, 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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