Kinetic Isotope Effects for Acyl Transfer from *p*-Nitrophenyl Acetate to Hydroxylamine Show a pH-Dependent Change in Mechanism

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Abstract: Kinetic isotope effects were measured for the acyl transfer reaction from *p*-nitrophenyl acetate to hydroxylamine at pH 6.0 and 12.0. The isotope effects measured and their values at pH 6.0 and 12.0, respectively, are as follows: leaving group oxygen-18 isotope effect, 1.0310 and 1.0074; the carbonyl oxygen-18 isotope effect, 1.0082 and 1.0008; the β-deuterium isotope effect, 0.9644 and 0.9516; the carbonyl carbon-13 isotope effect, 1.0287 and 1.0337; the nitro group nitrogen-15 isotope effect, 1.0009 and 1.0011. The solvent deuterium isotope effect is 1.4 at pH 7.5 and 0.85 at pH 12.0. The leaving group oxygen-18 isotope effect was measured as a function of pH between 6.0 and 12.0 and was found to have an inflection at pH 10.0 between the plateau regions at high and low pH. The results are consistent with rate limiting breakdown of an intermediate for the low pH reaction and attack of hydroxylamine anion with concerted expulsion of nitrophenolate ion at high pH.

Introduction

In 1958, Jencks[1] discovered that hydroxylamine cleaves *p*-nitrophenyl acetate (PNPA) with rapid formation of the ester *O*-acylhydroxylamine, followed by slow formation of the hydroxamic acid. Release of nitrophenol is much faster than for reactions with oxygen nucleophiles of similar p*K* and is ten million times faster than hydrolysis at neutral pH.[1] Jencks postulated a stepwise mechanism for formation of the ester, but the rate limiting step was undetermined. The large difference in rate indicates that the neighboring amine group in hydroxylamine plays a role in its increased reactivity, and three mechanisms were proposed for attack of the oxygen atom on PNPA:[1] (I) attack of zwitterionic hydroxylamine with hydrogen bonding between the protonated amino group and the carbonyl oxygen; (II) attack by uncharged hydroxylamine with hydrogen bonding between the neutral amine group and the carbonyl oxygen; or (III) attack of hydroxylamine with concerted intramolecular general base assistance by the amine group and hydrogen bonding by the partially protonated amine to the carbonyl oxygen. Interaction of the amine with the carbonyl group was also postulated to occur in the breakdown of the tetrahedral intermediate.

To elucidate the rate limiting step and the transition state structure of that step, kinetic isotope effects on this reaction have been measured. The isotope effects measured in this study, shown graphically in Figure 1, were those at the β-deuterium position in the acyl group (^{D}k), the carbonyl carbon atom (^{13}k), the carbonyl oxygen ($^{18}k_{carbonyl}$), leaving group phenolic oxygen ($^{18}k_{1g}$), and leaving group nitrogen (^{15}k) positions of PNPA at pH 6.0 and 12.0. In addition the $^{18}k_{1g}$ isotope effect was measured as a function of pH to probe the effect of pH on the mechanism. The results of these experiments are compared with previously determined isotope effects for PNPA hydrolysis and for acyl transfer from PNPA to oxygen and to nitrogen nucleophiles.

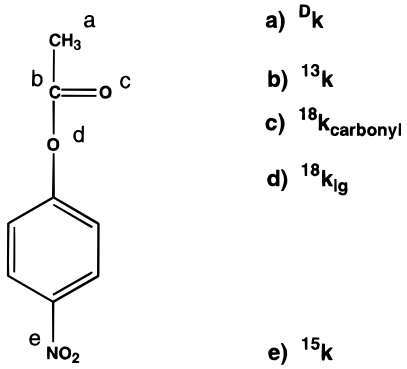

Figure 1. The *p*-nitrophenyl acetate substrate showing the positions where isotope effects were measured.

Experimental Section

Natural abundance PNPA was purchased from Aldrich and recrystallized from hexanes before use. [1-^{13}C]Acetyl chloride (99%) was from Aldrich. [^{15}N, *phenolic*-^{18}O]PNPA, [^{15}N,*carbonyl*-^{18}O]PNPA, [^{15}N,β-D_3]PNPA, and [^{14}N]PNPA were prepared as previously described.[2]

Preparation of [^{15}N,1-^{13}C]PNPA. [1-^{13}C]Acetyl chloride (61 μL, 0.86 mmol) was added to 3 mL of chloroform and 100 mg (0.7 mmol) of [^{15}N]-*p*-nitrophenol, followed by 87.8 mg (0.7 mmol) of 4-(dimethylamino)pyridine. After 3 h at room temperature, the mixture was partitioned between 0.05 N HCl and methylene chloride. The aqueous layer was washed again with methylene chloride, the combined organic layers were dried over magnesium sulfate, and the solvent was removed by rotary evaporation. The product was purified by flash chromatography, with elution by equal parts methylene chloride/cyclohexane, and recrystallized from hexanes. Analysis by mass spectrometry showed it to consist of 97% ^{15}N,^{13}C compound, with the remainder ^{15}N,^{12}C.

Isotope effects were measured by the remote label technique, using a ^{15}N label in the nitro group as a reporter for an ^{18}O, ^{13}C, or deuterium isotope effect.[2] In this technique substrate is synthesized with labels at two positions, one at the site of chemical interest and the other at a position which lends itself to facile isolation and isotopic measurement (the remote label). This double labeled material is mixed with substrate containing only the natural abundance of ^{18}O, ^{13}C, or deuterium in the

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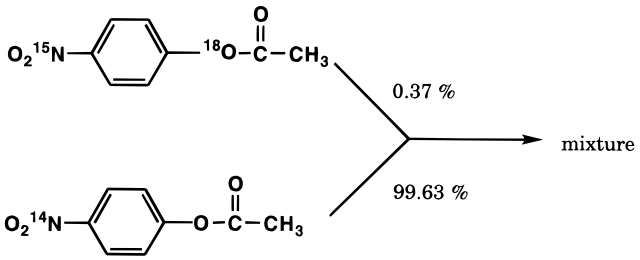

Figure 2. A schematic representation of the remote label method, as applied to the measurement of the $^{18}k_{1g}$ isotope effect with the nitrogen atom as the remote label.

position of interest, but depleted material in the remote label position (see Figure 2). The mixing ratio is such that the natural abundance of ^{15}N is restored in the remote label position. When this mixture is used in an experiment, the observed isotope effect is the product of that in the position of interest and that due to ^{15}N substitution. The ^{15}N isotope effect is determined with substrate containing only the natural abundance of isotopes in all positions, and the ratio of the two observed isotope effects is the desired one in the position of interest. With pNPP the nitrogen atom serves as a convenient remote label, as previously described.[2] The observed ^{18}O, ^{13}C, or deuterium isotope effects were corrected for the ^{15}N effect and for incomplete levels of isotopic incorporation in the starting material. All isotope effects were measured by the competitive method, using a Finnegan Delta E isotope ratio mass spectrometer to determine $^{15}N/^{14}N$ mass ratios.

Kinetic Isotope Effect Reactions. General Methods. Reactions were performed with 50 mL of 2 mM PNPA solution. After partial reaction, the reactions were stopped by partitioning the reaction mixture between water and methylene chloride. Both residual PNPA and *p*-nitrophenol were taken into the organic layer. These were separated by adding CHES buffer (1 M, pH 9.0) and washing twice with 40 mL of chloroform or methylene chloride. Control experiments showed that the *p*-nitrophenolate and PNPA were quantitatively separated when the pH was 9.0 or above with the phenolate remaining in the aqueous layer. The PNPA in the organic phase was completely hydrolyzed by treatment with 50 mL of 100 mM NaOH and rotary evaporation to remove the organic solvent.

The resulting aqueous *p*-nitrophenolate solutions were acidified to below pH 4 with hydrochloric acid, and the *p*-nitrophenol was extracted into three 50-mL volumes of ether. The ether layers were combined and dried over magnesium sulfate. The ether was evaporated and the *p*-nitrophenol was purified by vacuum sublimation at 90 °C for 15 min. Molecular nitrogen was isolated from nitrophenol by combustion and subjected to isotope ratio analysis as previously described.[2]

Isotope Effects at Low pH. Reactions were performed at 0 °C. To a 53-mL solution which was 2 mM in isotopically labeled or natural abundance PNPA and 100 mM in buffer (acetate, MES, HEPES, CHES, or CAPS), 1 mL of 1.4 M hydroxylamine hydrochloride (Fisher, 26 mM final) neutralized with NaOH was added in an ice bath (measured at 0 °C). At pH 6 the half-life was about 4 min. The reactions were stopped by separation into 60 mL of methylene chloride, which quantitatively removed unreacted PNPA into the organic layer and, at pH below 9, some *p*-nitrophenol as well. To separate these two components, the aqueous phase was adjusted to pH 9 with CHES buffer, the organic phase added back, and the mixture shaken again.

Isotope Effects at pH 12. All reactants were at 0 °C before use. PNPA (50 mL, 2 mM) was quickly mixed with 50 mL of 20 mM NaOH/20 mM hydroxylamine solution. The reaction was stopped by separation with 100 mL of methylene chloride after 1 half-life of 15 s.

Data Analysis. The kinetic isotope effects were calculated by using the nitrogen isotopic ratios from the product at partial reaction (R_p), from the remaining substrate (R_s), and from the isotopic ratio in the starting material (R_0). Equation 1 was used to calculate the observed isotope effect from the isotopic ratios of the product and starting material at known fractions of reaction, *f*. Equation 2 was used to calculate the observed isotope effect from the isotopic ratios of residual substrate and the starting material.

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$$\text{isotope effect} = \log(1 - f)/\log(1 - f(R_p/R_0)) \quad (1)$$

$$\text{isotope effect} = \log(1 - f)/\log((1 - f)(R_s/R_0)) \quad (2)$$

The oxygen-18, β-deuterium, and carbon-13 isotope effects were measured with use of mixed double-labeled substrates. These experiments yield an observed isotope effect, which is the product of the effect due to the nitrogen-15 and that due to the oxygen-18, carbon-13, or three deuteriums. The observed isotope effects from these experiments were corrected for the nitrogen-15 effect and for incomplete levels of isotopic incorporation in the starting material as previously described.[3]

Solvent Isotope Effects. The solvent isotope effects were measured at 20 °C. At pH 7.5, the solvent deuterium isotope effect was measured by direct comparison of rates in H_2O and D_2O. Each reaction mixture was 0.5 M in HEPES buffer (pH or pD 7.5), 0.033 mM PNPA, and 4 mM hydroxylamine. HEPES and NH_2OH were exchanged in D_2O prior to use in measuring the rate in D_2O.

At pH 12, the solvent deuterium isotope effect was measured by the proton inventory method with an OLIS rapid mixing, scanning spectrophotometer. A two-syringe system was used with one syringe filled with a solution containing 10 mM NaOH, 10 mM NaOD, 0.05 M hydroxylamine, and 0.05 M deuterated hydroxylamine; the second syringe was filled with 0.33 mM PNPA and H_2O, D_2O, or 50% H_2O/D_2O. One thousand scans per second were taken for 3 s, and the time course was fitted to a single exponential function. The plot of rate constant versus the fraction of H_2O was linear, and this plot was used to obtain the rate constants for reaction in pure H_2O and D_2O.

Acetohydroxamic acid was determined as the $FeCl_3$ complex. Three milliliters of sample were mixed with 0.1 mL of 10% $FeCl_3-6H_2O$ in 1 M HCl, and the absorbance was read at 540 nm and compared to that given by a standard solution of acetohydroxamic acid (Aldrich).

Results

Table 1 lists the isotope effects in the various positions of PNPA for reaction with hydroxylamine at pH 6.0 and 12.0 along with their standard errors. Each isotope effect reported is the mean of at least six independent determinations. The solvent deuterium isotope effect was 1.4 ± 0.1 at pH 7.5 and 0.85 ± 0.01 at pH 12.

The pH dependence of $^{18}k_{1g}$ is shown in Figure 3. The data were fitted to the following equation: $\log Y = \log[(Y_L + Y_H(K/H))/(1 + K/H)]$, where Y_L is the isotope effect at low pH and Y_H is the isotope effect at high pH. The fit to the data yielded the curve shown in the figure and values of $Y_L = 1.031 \pm 0.0006$, $Y_H = 1.008 \pm 0.001$, $pK = 10.1 \pm 0.1$.

Acetohydroxamic acid accounted for less than 4% of the products for the reactions at both high and low pH. Control experiments showed that hydroxide ion attack on PNPA at pH 12 accounted for less than 10% of the reaction observed. At higher pH values the hydroxide reaction becomes too competitive with the reaction with hydroxylamine to obtain reliable isotope effect data.

The isotope effects for the hydroxylamine reaction may be compared to the previously determined[2] isotope effects for acyl transfer from PNPA to the oxygen nucleophiles hydroxide and hexafluoroisopropoxide and to the nitrogen nucleophile methoxyethylamine, which are also shown in Table 1.

Discussion

PNPA cleavage by hydroxylamine is 400 times faster at pH 12 than at pH 6, and the magnitude of $^{18}k_{1g}$ is decreased from 1.0310 at pH 6 to 1.0074 at pH 12, with an inflection at pH 10 (Figure 3). This suggests a change in mechanism occurs upon the change from attack of neutral hydroxylamine at low pH to attack of the oxyanion at high pH (the pK_a of hydroxylamine is 13.7). The failure to observe significant amounts of the product

Table 1. Isotope Effects for Reaction of Hydroxylamine with PNPA at pH 6.0 and 12.0

pH	^{15}k	$^{18}k_{1g}$	$^{18}k_{carbonyl}$	$^{D}k^{a}$	^{13}k
6	1.0009 ± 0.0003	1.0310 ± 0.0010	1.0082 ± 0.0005	0.9644 ± 0.0002	1.0287 ± 0.0047
12	1.0011 ± 0.0007	1.0074 ± 0.0013	1.0008 ± 0.0014	0.9516 ± 0.0003	1.0337 ± 0.0011
nucleophile[b] (pK_a)	^{15}k	$^{18}k_{1g}$	$^{18}k_{carbonyl}$	$^{D}k^{a}$	^{13}k
OH^- (15.7)	1.0002 ± 0.0001	1.0135 ± 0.0007	1.0039 ± 0.0003	0.9562 ± 0.0008	1.038 ± 0.001
phenolate (9.9)	1.0009 ± 0.0002	1.0182 ± 0.0009	1.0039 ± 0.0008	0.9617 ± 0.0010	not measured
$(CF_3)_2CHO^-$ (9.7)	1.0010 ± 0.0002	1.0210 ± 0.0010	1.0058 ± 0.0006	0.9481 ± 0.0030	1.029 ± 0.001
methoxyethylamine	1.0011 ± 0.0001	1.0330 ± 0.0007	1.0064 ± 0.0003	0.9682 ± 0.0010	1.028 ± 0.0017

[a] Isotope effect due to three deuterium atoms in the acyl group. [b] The isotope effects ^{15}k, $^{18}k_{1g}$, $^{18}k_{carbonyl}$, and ^{D}k for reactions with hydroxide, phenolate, hexafluoroisopropoxide and methoxyethylamine are from ref 2.

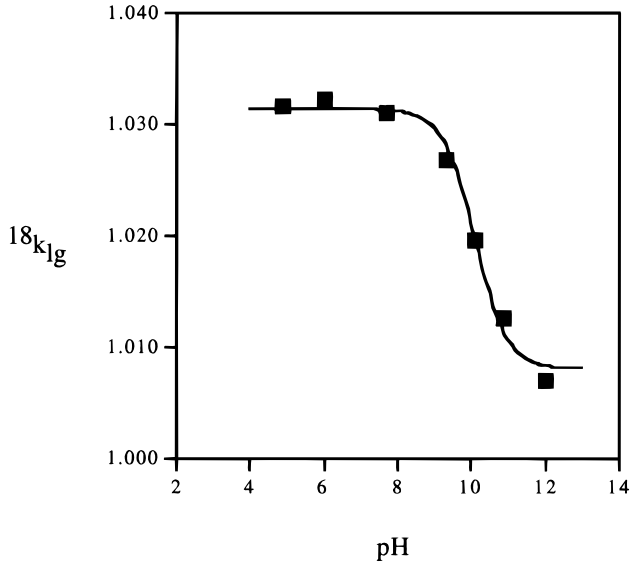

Figure 3. pH dependence of $^{18}k_{1g}$ for acyl transfer from PNPA to hydroxylamine. For details see the Results section.

of amine attack, acetohydroxamic acid, rules out a significant contribution from this alternate nucleophile reaction at low or high pH.

The $^{18}k_{1g}$ isotope effect will be sensitive to the nature of the rate-determining step. The other isotope effects should also be sensitive to such changes in mechanism, but the larger magnitude of the $^{18}k_{1g}$ effect and its more straightforward dependence upon the nature of the rate-limiting step make it the best tool for this purpose. If formation of the intermediate is rate limiting, this isotope effect will be a secondary one and its magnitude will thus be small, and will reflect the change in hybridization of the carbonyl carbon atom in the transition state for nucleophilic attack. The isotope effects in the acyl group, $^{18}k_{carbonyl}$ and ^{D}k, should exhibit magnitudes reflective of the degree of loss of the carbonyl π-bond in the transition state. For a late transition state values of about 1.025 for $^{18}k_{carbonyl}$ and 0.89 for ^{D}k are expected.[4]

If the breakdown of a tetrahedral intermediate is the rate-limiting step then $^{18}k_{1g}$ will be a primary isotope effect, of a large magnitude that will be sensitive to the extent of bond cleavage in the transition state. Similarly, a significant value for ^{15}k should be observed, reflecting delocalization of charge into the nitrophenyl ring of the leaving group. The $^{18}k_{carbonyl}$ and ^{D}k isotope effects should be reduced from their values for rate-limiting attack, as with rate-limiting breakdown these will be the product of opposing effects, the equilibrium effects for formation of the intermediate, and the kinetic effects for its breakdown.

In a concerted mechanism, $^{18}k_{1g}$ will again be a primary effect and large isotope effects should be exhibited in the leaving group. The values for $^{18}k_{carbonyl}$ and ^{D}k would be expected to be small, reflective of whatever tetrahedral character in the carbonyl group is present in the transition state.

Reaction at pH 12.0. Acyl transfer from PNPA in water to oxygen nucleophiles has been shown to be concerted by evidence from linear free energy relationships,[5] isotope effects,[2] and theoretical treatment with Marcus theory.[6] If acyl transfer from PNPA to the hydroxylamine anion proceeded by a tetrahedral mechanism, the intermediate should partition nearly exclusively forward by expulsion of *p*-nitrophenolate, a much better leaving group than the hydroxylamine oxide anion. The isotope effects would then reflect formation of the intermediate. By comparing the set of isotope effects for concerted acyl transfer from PNPA to hydroxide, phenolate and hexafluoroisopropoxide nucleophiles, it becomes evident that the acyl transfer to hydroxylamine anion is also concerted, with a very early transition state. There is minimal change in bonding to the carbonyl oxygen ($^{18}k_{carbonyl} = 1.0008$) although the β-deuterium isotope effect ($^{D}k = 0.9516$) indicates a significant loss of hyperconjugation in the transition state. Bond cleavage to the leaving group is only slightly advanced ($^{18}k_{1g} = 1.0074$), with a moderate level of charge delocalization through the nitrophenol ring ($^{15}k = 1.0011$). These latter two values can be compared with the equilibrium isotope effects for the equilibrium between PNPA and *p*-nitrophenolate ion, which are 1.0277 for $^{18}k_{1g}$ and 1.0016 for ^{15}k.[2] The carbonyl carbon-13 isotope effect remains large and normal, indicating that this is a primary isotope effect and ruling out alternate rate limiting steps such as desolvation of the nucleophile.

The inverse solvent D_2O isotope effect of 0.85 will mainly result from equilibrium isotope effects on the formation of free hydroxylamine anion (eq 3). The formation of water (fractionation factor = 1) from hydroxide (fractionation factor = 0.48[7]) will contribute an inverse isotope effect that will be partially offset by normal isotope effects from loss of the H−N−O−H torsional modes in ionized hydroxylamine.

$$OH^- + NH_2OH \rightleftharpoons NH_2O^- + H_2O \qquad (3)$$

On the basis of the reduction in the ^{18}O isotope effects, the transition state appears to be earlier (more substrate-like) for acyl transfer to hydroxylamine anion than for transfer to hydroxide, even though the pK_a of water is 15.8 and that of hydroxylamine is 13.7.[8] Anomalous nucleophilic behavior of hydroxide has been previously noted in the correlation of

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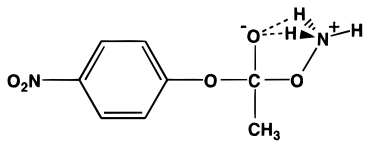
nucleophile pK_a with the rate of PNPA hydrolysis[9] and has been attributed to a high barrier to desolvation. On the basis of isotope effect data, Marlier showed that hydroxide cleaves methyl formate by attack of a water molecule hydrating the hydroxide, with the latter acting as a general base.[10]

Reaction at pH 6.0. Acyl transfer to neutral hydroxylamine must involve proton transfer from its oxygen or nitrogen atoms to water, to the carbonyl oxygen atom, or to the leaving group oxygen atom. This reaction may well proceed via a stable intermediate since such a species would be neutral rather than anionic. In relation to the equilibrium phenolic oxygen-18 isotope effect of 1.0277 for the PNPA−*p*-nitrophenolate equilibrium,[2] the observed $^{18}k_{1g}$ of 1.0310 is large and is most likely due to bond breaking in the rate limiting decomposition of an intermediate. The observed β-deuterium and carbonyl ^{18}O isotope effects are then products of the equilibrium isotope effect on formation of the intermediate and the kinetic isotope effect on its decomposition. The overall pattern of isotope effects for this reaction is similar to that for the reaction with methoxyethylamine (Table 1), which is believed to proceed through rate-limiting breakdown of a zwitterionic tetrahedral intermediate.[11] The normal solvent deuterium isotope effect of 1.4 may arise from deprotonation of hydroxylamine during the breakdown of the tetrahedral intermediate.

The question of the role of the amine group in catalysis of the reaction at neutral pH can be reconsidered in the light of the present results. The intramolecular catalysis provided by the amine group probably lowers the barrier to the addition step. Intramolecular base catalysis by the amine, possibly via an intervening water molecule, is a likely pathway due to the proximity of the amine to the nucleophilic hydroxyl. The concurrent polarization of the carbonyl group by hydrogen bonding with the amine is also likely but is most likely to occur as a bifurcated, three-center hydrogen bond. This is because studies of the geometry of hydrogen bonds which involve a single hydrogen atom indicate that in most cases the hydrogen bond is linear, or nearly so.[12] For this reason the vast majority of intramolecular hydrogen bonds involve six-membered rings, and those involving five-membered rings are relatively rare. By contrast bifurcated internal hydrogen bonds involving five-membered rings have been found in methyl hydrazine carboxylate[13] and in glycine methyl ester.[14] These compounds are very similar in structure to the transition state for attack of hydroxylamine on PNPA, and indicate that if electrophilic assistance via internal hydrogen bonding occurs the interaction will most likely resemble such a bifurcated hydrogen bond between two amino hydrogens and the carbonyl oxygen atom. Significant covalent proton transfer to the carbonyl oxygen can be ruled out since this should produce an inverse $^{18}Ok_{carbonyl}$ isotope effect, which is not observed.

The most likely scenario for the reaction at low pH is attack by the hydroxyl group of neutral hydroxylamine with concurrent proton transfer to the neighboring nitrogen atom, giving the zwitterionic intermediate **1**. The nucleophile is easily expelled by the reverse process, and thus breakdown of the intermediate is rate limiting.



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Conclusions

The isotope effect data at pH 6.0 are consistent with the formation of *O*-acylhydroxylamine proceeding via rate-limiting breakdown of the zwitterionic tetrahedral intermediate formed from attack of uncharged hydroxylamine on PNPA. The amine group may contribute to reactivity by acting as an intramolecular general base, and possibly also by electrophilic polarization of the carbonyl group. The transition state for breakdown of the intermediate exhibits considerable bond cleavage to the leaving group. At pH 12.0, reaction proceeds via concerted attack of the anion of hydroxylamine and departure of *p*-nitrophenolate, with a transition state that is earlier than that observed in the analogous reaction with hydroxide.

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