Isotope Effects on Enzyme-Catalyzed Acyl Transfer from *p*-Nitrophenyl Acetate: Concerted Mechanisms and Increased Hyperconjugation in the Transition State

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Abstract: To examine the mechanism of enzymatic acyl transfers from *p*-nitrophenyl acetate (PNPA), isotope effects were measured for the reaction of PNPA with chymotrypsin, carbonic anhydrase, papain, and *Aspergillus* acid protease. The isotope effects were measured at the β-deuterium (^{D}k), carbonyl carbon (^{13}k), carbonyl oxygen ($^{18}k_{carbonyl}$), leaving group phenolic oxygen ($^{18}k_{lg}$), and leaving group nitrogen (^{15}k) positions. ^{D}k ranged from 0.982 ± 0.002 to 0.999 ± 0.002. ^{13}k ranged from 1.028 ± 0.002 to 1.036 ± 0.002. $^{18}k_{carbonyl}$ ranged from 1.0064 ± 0.0003 to 1.007 ± 0.001. $^{18}k_{lg}$ ranged from 1.141 ± 0.0002 to 1.330 ± 0.0007. ^{15}k ranged from 0.9997 ± 0.0007 to 1.0011 ± 0.0002. Uncatalyzed acyl transfer from PNPA to oxygen and sulfur nucleophiles proceeds by a concerted mechanism. All of the enzymatic reactions showed isotope effects consistent with a concerted mechanism like that seen in uncatalyzed aqueous reactions, but exhibited smaller inverse β-deuterium isotope effects than seen in the nonenzymatic aqueous reactions. This phenomenon may be explained by greater hydrogen bonding or electrostatic interaction with the ester carbonyl group in enzymatic transition states relative to nonenzymatic aqueous transition states. Quantum mechanical calculations were used to estimate the magnitude of changes in hyperconjugation and C−H bond order due to protonation of a carbonyl oxygen.

Introduction

A major contribution to enzymatic catalysis is the selective stabilization of transition states. Kinetic isotope effects can illuminate how an enzyme changes the electronic and nuclear structure of the enzymatic transition state relative to nonenzymatic transition states in solution. Here we apply a system for accurately and precisely measuring isotope effects in several positions for the acyl transfer from *p*-nitrophenyl acetate to observe enzyme induced changes in transition state force fields. By comparing the isotope effects on enzyme-catalyzed reactions with previously measured isotope effects of similar nonenzymatic reactions in solution,[1] we may gain insight into mechanisms of enzymatic catalysis. 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 ($^{18}k_{lg}$), and leaving group nitrogen (^{15}k) positions. The isotope effects were measured by the competitive method and are therefore effects on *V*/*K*. Thus for each of the enzymes examined the isotope effects are sensitive only to the transition state for the acyl transfer from the PNPA substrate, even though hydrolysis of an acyl enzyme intermediate is the rate-limiting step for V_{max} in the mechanisms of some of the enzymes examined.

The existence of concerted acyl transfer from esters with good (i.e., aryl) leaving groups has been a matter of some controversy.[2] Previous work in this laboratory has shown that kinetic isotope effects for acyl transfers from *p*-nitrophenyl acetate (PNPA) to oxygen and sulfur nucleophiles support a concerted mechanism.[1] In contrast, by extension of the evidence from isotopic exchange of ^{18}O from water into nonactivated esters in favor of stable intermediates, enzyme-catalyzed acyl transfers from PNPA and other activated esters have been postulated to proceed via stable tetrahedral intermediates.[3] In the present work, kinetic isotope effects were measured to test the hypothesis that oxyanion binding sites of enzymes could change the reaction mechanism by binding to and stabilizing a tetrahedral intermediate, resulting in a change to a stepwise mechanism for this activated ester.

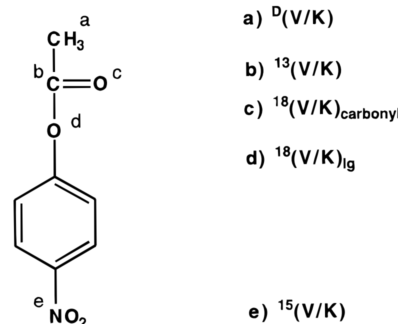

Figure 1. The *p*-nitrophenyl acetate substrate showing the positions where isotope effects were measured.

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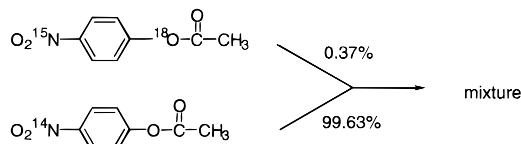

Figure 2. Diagrammatic representation of the preparation of the mixture used to measure ^{18}O, ^{13}C, and β-deuterium isotope effects in PNPA (in this example $^{18}k_{lg}$) with the remote label method, where the nitrogen atom in the nitro group serves as the remote label.

β-Deuterium isotope effects are particularly useful in analyzing reactions of carbonyl compounds. The inverse β-deuterium isotope effects associated with nucleophilic addition to a carbonyl carbon are due to a loss of hyperconjugation and a stiffening of the β-CH bonds.[4] There is a smaller contribution to the isotope effect from restricted methyl rotation.[5] The equilibrium isotope effect of 0.87 per three deuteriums for acetaldehyde hydration may be taken to be the maximum effect on rehybridization of the carbonyl group.[6] Hyperconjugation arises through orbital overlap and electron donation from the σ CH bonding orbital to the carbonyl π^* antibonding orbital. A hybridization change due to nucleophilic addition to the carbonyl group will destroy this hyperconjugation and cause an inverse isotope effect, while an electrophilic interaction with the carbonyl oxygen should increase the Lewis acidity of the carbonyl π^* orbital, increase hyperconjugation, and lead to a normal β-deuterium isotope effect. Hyperconjugation effects are often attributed to changes in positive charge on the carbonyl carbon; this is an incomplete description of the phenomenon.[7] The extent of orbital overlap and the populations of the bonding and antibonding orbitals should also be considered. These factors may become relevant for geometrically constrained systems or otherwise unstable configurations such as transition states. A useful way to model these effects is with the natural bonding orbital (NBO) and natural resonance theory (NRT) algorithms of Weinhold et al.[8] We present calculations of the extent of hyperconjugation in acetaldehyde to estimate the magnitude of hyperconjugation.

Experimental Section

Natural abundance PNPA was purchased from Aldrich and recrystallized from hexanes before use. Chymotrypsin A (bovine pancreas) was from Boehringer Mannheim. Papain, carbonic anhydrase, and *Aspergillus* acid protease were from Sigma. [1-^{13}C] acetyl chloride (99%) was from Aldrich. [^{15}N,*phenolic*-^{18}O]PNPA, [^{15}N,*carbonyl*-^{18}O]PNPA, [β−D_3,^{15}N]PNPA, and [^{14}N]PNPA were prepared as previously described.[1]

Remote Labeled PNPA. PNPA was synthesized with a ^{15}N label in the nitro group as a reporter for a ^{18}O, ^{13}C, or deuterium isotope effect. In this method the aforementioned doubly labeled substrate is mixed with [^{14}N]PNPA (that is, containing nitrogen depleted in ^{15}N). Figure 2 shows a schematic representation of this process for the measurement of the isotope effect $^{18}k_{lg}$. The mixing ratio was adjusted so that the nitrogen was at natural abundance in order to minimize error due to contamination by air and maximize the sensitivity for measurement of isotope effects. The secondary nitrogen isotope effects were measured with use of the natural abundance compound. All isotope effects were measured by the competitive method, using a Finnigan Delta E isotope ratio mass spectrometer to determine ^{15}N/^{14}N mass ratios.

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.

Kinetic Isotope Effect Determinations. General Methods. Reactions were performed with 50 mL of 2 mM PNPA solution, to which buffer and the appropriate enzyme were added. After partial reaction, reactions were stopped and, if necessary, the bulk of the protein was removed as detailed below. Separation of *p*-nitrophenol and PNPA was accomplished by washing twice with 40 mL of chloroform or methylene chloride. Control experiments showed that the *p*-nitrophenolate and remaining PNPA were quantitatively separated when the pH was above 9.0. The residual PNPA in the organic phase was completely hydrolyzed by treatment with 50 mL of 100 mM NaOH followed by removal of the organic solvent by rotary evaporation.

The resulting aqueous *p*-nitrophenolate solutions were acidified to below pH 4 with 5 M 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.[9]

General Conditions for Isotope Effect Reactions. The reactions were found to be dependent on enzyme concentrations and independent of buffer concentration. The concentration of PNPA was 2 mM in each reaction. All reactions were carried out at 24 °C. Sufficient quantities of enzyme were used so that uncatalyzed hydrolysis of PNPA was less than 1% of the enzymatic rate.

Reactions with Chymotrypsin. Lyophilized enzyme (75 mg) was added to 52.5 mL of 2 mM PNPA with 50 mM HEPES, pH 7.0. After 1 half-life of approximately 90 min, the reaction was stopped by raising the pH to 9.0 with 1 M CHES, pH 10, and extraction of PNPA with chloroform. The aqueous samples were heated to 75 °C for 10 min, and 10 g of hydrated magnesium sulfate was added. The samples were cooled on ice and centrifuged for 5 min at 3000 g to remove the precipitated protein.

Because of the relatively large quantity of enzyme needed, an inactivation study was done to ensure that the reaction was catalyzed by the chymotrypsin active site. *N*-Tosylphenylalanine chloromethyl ketone (TPCK) (250 μL of 23 mM) in acetonitrile or acetonitrile alone was added to separate 20 mL solutions of chymotrypsin (1 mg/mL). Enzymatic activity was assayed with use of acetyltryptophan ethyl ester. After 2.5 h, activity was reduced by greater than 95% in the sample treated with TPCK as compared to untreated enzyme. Hydrolysis of PNPA at pH 9 showed a similar ratio of rates with treated versus uninhibited chymotrypsin.

Reactions with Carbonic Anhydrase. Five milliliters of carbonic anhydrase solution (0.5 mg/mL) was added to 52.5 mL of 2 mM PNPA and 50 mM CHES, pH 9.0. The reaction half-life was 10 min. The reaction was stopped by separation with methylene chloride and both aqueous and organic layers were filtered with Whatman #1 filter paper.

Reactions with Papain. Enzyme solution (2.5 mL, 28 mg/mL) was added to 52.5 mL of 2 mM PNPA with 0.5 mM dithiothreitol, 0.5 mM EDTA, and 50 mM MES buffer, pH 6.0. The reaction was stopped by addition of 4 mL of 20% acetic acid after a half-life of 3 h. The nitrophenol and residual PNPA were extracted by washing three times with 50 mL of ether. The ether was removed by rotary evaporation, and the residue was resuspended in 50 mL of 200 mM CHES, pH 9.5.

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Table 1. Isotope Effects for Acyl Transfers from PNPA

enzyme/nucleophile	^{15}k	$^{18}k_{lg}$	$^{18}k_{carbonyl}$	$^{D}k^{a}$	^{13}k
chymotrypsin	1.0011 ± 0.0002	1.0204 ± 0.0003	1.0065 ± 0.0001	0.982 ± 0.002	1.030 ± 0.002
carbonic anhydrase	1.0005 ± 0.0002	1.0255 ± 0.0007	1.0075 ± 0.0004	0.999 ± 0.002	1.028 ± 0.002
aspergillus acid protease	0.9997 ± 0.0007	1.0141 ± 0.0002	1.007 ± 0.001	0.986 ± 0.002	1.036 ± 0.002
papain	1.0011 ± 0.0001	1.0330 ± 0.0007	1.0064 ± 0.0003	0.9946 ± 0.0005	1.034 ± 0.002
$(F_3C)_2CHO^-$ [b]	1.0010 ± 0.0002	1.0210 ± 0.0003	1.0065 ± 0.0001	0.948 ± 0.003	1.0294 ± 0.001
3-mercaptopropionate[b]	1.0003 ± 0.0001	1.0172 ± 0.0009	1.0117 ± 0.0004	0.9765 ± 0.0006	1.0343 ± 0.001

[a] Isotope effects resulting from substitution of all three β-hydrogen atoms by deuterium. [b] Measured previously.[1]

The nitrophenolate and PNPA were then immediately separated by partitioning with 50 mL of methylene chloride. The reaction rate was found to depend linearly on the enzyme concentration, and no significant reaction was observed for a control experiment containing PNPA, MES buffer, dithiothreitol, and EDTA.

Reactions with *Aspergillus* Acid Protease. Enzyme (35 mg) was added to 53 mL of PNPA with 57 mM formate buffer, pH 3. This pH was chosen to ensure that no catalysis by adventitious contaminating proteases occurred. The half-life of the reaction was about 7 h. Methylene chloride was used for the separation of product and residual substrate.

The pH dependence of V/K for the *aspergillus* acid protease was determined by producing Lineweaver−Burk plots at a range of pH values from 2.6 to 6.9 with PNPA concentrations of from 0.7 to 2.6 mM at 23 °C. The buffers used were formate, acetate, MES, and HEPES at concentrations of 100 mM. Reactions were initiated by addition of 100 μL of an 8.5 mg/mL solution of the enzyme.

Data Analysis. The kinetic isotope effects were calculated using the 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_o). 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.

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

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

The oxygen-18, β-deuterium, and carbon-13 isotope effects were measured by using 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 the incomplete levels of isotopic incorporation in the starting material as previously described.[10]

Calculations. Geometry optimizations and electronic structure computations were performed with GAUSSIAN92 or GAMESS at the RHF/6-31G* level. Natural bond orbitals, natural bond orders, and perturbation energies and resonance contributions were calculated with the NBO and NRT programs.[11] For the acetaldehyde/ammonium ion complex, the ammonium was constrained to be tetrahedral and the non-hydrogen-bonded hydrogens were constrained to be equidistant.

Results

The measured isotope effects and their standard errors are presented in Table 1. At least six independent determinations of each isotope effect were carried out. The ^{18}O, β-deuterium, and ^{13}C isotope effects have been corrected for the ^{15}N effect and for incomplete isotopic incorporation. Table 1 lists the isotope effects for the enzyme catalyzed reactions and those for two previously determined nonenzymatic aqueous reactions for comparison.

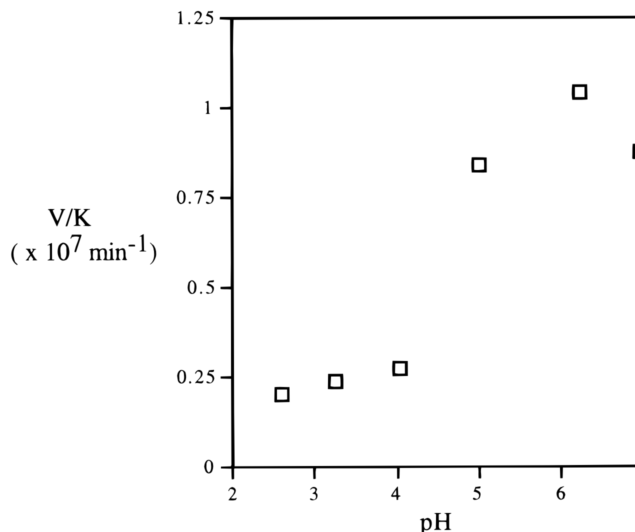

Figure 3. V/K pH profile for the reaction of *Aspergillus* acid protease with PNPA.

The V/K pH rate profile for *Aspergillus* acid protease catalyzed hydrolysis of PNPA is shown in Figure 3. Although the maximum V/K is higher than that for amide hydrolysis (approximately 3), the enzyme is active at pH 2.

Some parameters from the quantum mechanical calculations which are relevant to hyperconjugation are listed in Tables 2 and 3. Figure 5 shows contour plots of the overlap between one of the hyperconjugative hydrogens and the π^* orbital. The greater orbital overlap in the protonated acetaldehyde than in acetaldehyde is quantified as the overlap integral $\langle sCH|\pi^*CO\rangle$ in Table 3.

Previous attempts to use ab initio calculations to optimize the geometries of the products resulting from the addition of hydrogen sulfide to formamide and to formaldehyde failed to locate stable species such as those that result from addition of hydroxide.[12] A similar phenomenon was observed in this work for the acetaldehyde adduct of methanethiolate ion, which has a higher pK than hydrogen sulfide. To examine the role of sulfur d-orbitals in an addition complex, the carbon−sulfur bond distance was constrained to 1.65 Å (the C−O distance for the hydroxide adduct plus the difference in the covalent radii of oxygen and sulfur) and the geometry was optimized to the 6-31+G* level. No oxygen−sulfur bond order was found and no significant d-orbital occupation occurred.

Discussion

In a relevant previous study isotope effects were measured for the aqueous acyl transfer reactions of PNPA with several nucleophiles.[1] The data were used to distinguish between a two-step mechanism, proceeding by way of a tetrahedral intermediate, and a concerted mechanism, where nucleophilic attack and

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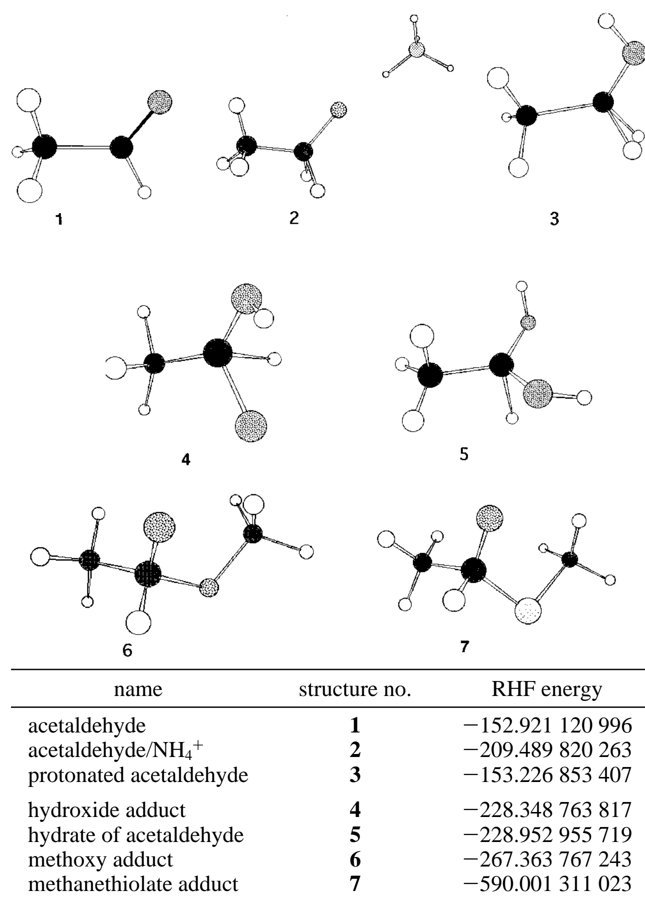
Table 2. 6-31+G* RHF Energies for 6-31+G* Optimized Geometries



name	structure no.	RHF energy
acetaldehyde	**1**	−152.921 120 996
acetaldehyde/NH_4^+	**2**	−209.489 820 263
protonated acetaldehyde	**3**	−153.226 853 407
hydroxide adduct	**4**	−228.348 763 817
hydrate of acetaldehyde	**5**	−228.952 955 719
methoxy adduct	**6**	−267.363 767 243
methanethiolate adduct	**7**	−590.001 311 023

leaving group departure occur in the same step. In a tetrahedral mechanism if the nucleophile has a significantly higher pK_a than the leaving group, the intermediate will partition essentially completely forward to products. In this case the isotope effects will reflect the first step of the mechanism, formation of the tetrahedral intermediate. The major change undergone in the substrate in this step is loss of the carbonyl π bond, and the bond to the leaving group is little affected. In a concerted mechanism, by contrast, bond cleavage to the leaving group occurs in the transition state and will be reflected in the isotope effects. The data were most consistent with a concerted mechanism, with bond cleavage to the leaving group approximately two-thirds complete in the transition state. The data also indicated partial loss of the carbonyl π bond, indicating that the transition states have some tetrahedral character.[1]

To interpret the enzymatic isotope effects in terms of a tetrahedral versus a concerted mechanism, one must make an assumption regarding the partitioning of a postulated tetrahedral intermediate. The enzymes in this study all possess nucleophiles having pK_a values well above the solution pK_a (7.1) of the leaving group. Unless the enzymes raise the pK_a values of the leaving group of the substrate (which would be anticatalytic), tetrahedral intermediates in the enzymatic reactions of PNPA ought to partition forward to products, and the isotope effects will then reflect formation of the intermediate.

^{13}k Isotope Effects. The carbonyl carbon isotope effects are primary ones, and will have contributions from bond formation and cleavage, from rehybridization, and from reaction coordinate effects, and so cannot be confidently analyzed in terms of specific bonding changes in the transition state. The large magnitude of this isotope effect makes it sensitive to the degree to which the acyl transfer step is rate-limiting for V/K. To the extent that another step such as substrate binding or a conformational change may be partially rate limiting, the isotope effects will be suppressed, and will not reflect the true structure of the transition state. The uncatalyzed reaction in solution should exhibit the full intrinsic isotope effects, and the large magnitude of the ^{13}k isotope effects indicates that chemistry is rate determining, as opposed to some other effect such as desolvation or diffusion. The similarity of the ^{13}k values for the enzymatic and for the uncatalyzed reactions demonstrates that for the enzymatic reactions studied the acyl transfer step is fully rate limiting.

Chymotrypsin and Carbonic Anhydrase. The $^{18}k_{lg}$, and $^{18}k_{carbonyl}$ isotope effects are similar to those observed in the aqueous reaction with the anion of hexafluoro-2-propanol, suggesting that the concerted nature of the acyl transfers from PNPA appears to be maintained in the enzyme-catalyzed reactions. The $^{18}k_{lg}$ values of 1.020 and 1.026 for the two enzymes may be compared to the corresponding equilibrium isotope effect for PNPA hydrolysis of 1.0281 and indicate a large degree of bond breaking to the leaving group in the enzymatic transition states. The $^{18}k_{carbonyl}$ isotope effects of 1.0065 and 1.0075 are lower than the estimated equilibrium isotope effect of 1.03 for formation of a tetrahedral intermediate[13] or the measured kinetic isotope effect of 1.024 for methanolysis of phenyl benzoate,[14] suggesting that the oxyanion hole does not add enough stabilization to the tetrahedral species to change the mechanism to a stepwise reaction.

However, in both the chymotrypsin and carbonic anhydrase reactions the β-deuterium isotope effects are substantially less inverse than their values in uncatalyzed reactions, such as that with the anion of hexafluoro-2-propanol (0.948). This isotope effect measures the loss of hyperconjugation as the sp^2 carbonyl carbon in the substrate becomes partially rehybridized in the partially tetrahedral transition state. The enzymatic results suggest that the oxyanion hole in chymotrypsin and the zinc cation in carbonic anhydrase polarize the carbonyl bond in the

Table 3. RHF 6-31G* NBO and NRT Calculations of Hyperconjugation in Acetaldehyde, Protonated Acetaldehyde, and the Acetaldehyde/Ammonia Complex

structure	% p hybrid[a] of σ CO bond(s)	pert. energy[b]	overlap integral[c]	ave CH bond order[d]	% resonance[e]	% polarization of π^* bond[f]
acetaldehyde H^+	71.8	23.49	0.1946	0.9847	3.98	83.44
acetaldehyde/NH_4^+	69.9	15.68	0.2129	0.9887	2.46	74.8
acetaldehyde	67.7	12.19	0.2021	0.9910	2.14	69.06
deprotonated hydrate of acetaldehyde	76.8, 71.2	NA[g]	NA[g]	0.9922	0.51	
hydrated acetaldehyde	77.7, 77.7	NA[g]	NA[g]	0.9936	0.33	

[a] The percent of p orbital character of the hybrid orbital at the carbonyl carbon. [b] The energy change on deletion of the $\sigma-\pi^*$ orbital overlap. [c] The overlap integral $\langle \sigma CH|\pi^* CO\rangle$ measures the extend of orbital overlap between the σ and π^* orbitals. [d] The average natural bond order for the three methyl CH bonds. [e] The percentage of the contribution of the resonance forms with broken methyl CH bonds using natural resonance theory. [f] $100|c_a|^2$ where c_a is the weight given to the carbon atom in forming the π^* NBO. [g] Not applicable.

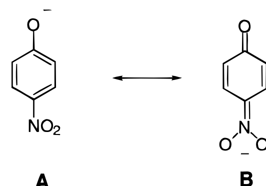


Figure 4. The magnitudes of the isotope effects ^{15}k and $^{18}k_{lg}$ will be affected by the relative contributions of the resonance forms shown (other resonance forms contribute, but only two are shown for purposes of illustration). In A, the full $^{18}k_{lg}$ isotope effect resulting from bond cleavage at oxygen will be expressed, and ^{15}k will be minimal as no charge is delocalized into the aromatic ring. Contributions from forms such as B will lower $^{18}k_{lg}$ due to compensatory C−O bond formation and will give rise to larger ^{15}k effects due to bonding changes at nitrogen.

transition state sufficiently to maintain more of the hyperconjugation present in the substrate, particularly in the carbonic anhydrase reaction. This is also consistent with the differences in the ^{15}k and $^{18}k_{lg}$ isotope effects in these reactions. The greater positive character of the carbonyl carbon in the transition state of the carbonic anhydrase reaction stabilizes the resonance form of the nitrophenolate leaving group with the charge on the phenolic oxygen atom. This results in a reduction of the charge delocalized into the aromatic ring (thus lowering ^{15}k) and lessening O−C bond order between oxygen and the ring carbon atom (thus increasing $^{18}k_{lg}$); see Figure 4.

Papain. Previous evidence indicates that the papain-catalyzed reaction of PNPA occurs by the same mechanism as for specific substrates.[15] The isotope effects for the papain-catalyzed reaction differ from previously determined effects for methyl 3-mercaptopropionate attack in water. The $^{18}k_{carbonyl}$ effect is 1.0064 for papain vs 1.0117 for the reaction with the aqueous thiolate, and the $^{18}k_{lg}$ values are 1.0330 and 1.0172, respectively. The oxyanion hole of papain may favor a more associative transition state over the expanded transition state seen in the nonenzymatic reactions with thiolates,[1] but the reaction remains concerted. Significant differences were found in the isotope effects for the uncatalyzed attack of thiolate versus oxyanion nucleophiles on PNPA.[1] However, the present data show that the transition state for the reaction of papain with PNPA more closely resembles those for enzymatic oxygen nucleophiles than the nonenzymatic acyl transfer to thiolates, and indicate much more similar transition state structures for the serine and cysteine proteases than for the corresponding oxygen versus sulfur nucleophilic acylation reactions in solution. Previous studies indicate that the nucleophile in the papain-catalyzed hydrolysis of PNPA is a thiolate, as is the case with specific substrates.[15]

Hupe and Jencks[16] have studied and discussed the differences between oxygen and sulfur nucleophiles in acyl transfer reactions from esters. Equilibrium constants for addition of thiol nucleophiles are about 1000 times larger than those for alcohols. Thiolates are 10 times more nucleophilic than phenolates of similar pK_a toward PNPA, and the β_{nuc} of 0.27 for thiolate anions is smaller than the β_{nuc} of 0.68 for phenolate nucleophiles. By using estimates of the "effective charge" at the transition state from Brønsted studies, Hupe and Jencks determined that there is an "effective charge" of −1.1 on the carbonyl oxygen for the transition state of phenolate attack but a charge of only −0.6 for the corresponding addition of thiolates. Hupe and Jencks presented two explanations for the differences between nucleophilic attack with oxygen and sulfur: back-bonding between a lone pair of the carbonyl oxygen and the d orbitals of the sulfur atom, or greater rehybridization around the carbonyl carbon for the thiolate reactions. Destruction of back-bonding by enzymatic hydrogen bonding to the carbonyl oxygen would explain why the isotope effects which describe the transition state for acyl transfer from PNPA to papain more closely resemble the isotope effects for the chymotrypsin-catalyzed reaction than the nonenzymatic reaction with methyl 3-mercaptopropionate. However, the NBO calculations presented here show no indication of any back-bonding interaction in the gas-phase addition of methanethiolate to acetaldehyde nor any charge reduction on the oxyanion.

The hypothesis of greater rehybridization in transition states of thiolate addition relative to phenoxide addition is consistent with the increased magnitude of $^{18}k_{carbonyl}$ for the nonenzymatic acyl transfers to methyl 3-mercaptopropionate compared with those observed for phenolate, but may contradict the less inverse ^{D}k for thiolate addition. Greater understanding of nonenzymatic reactions will help in the interpretation of the enzymatic mechanisms.

***Aspergillus* Acid Protease.** The $^{18}k_{lg}$ and ^{15}k isotope effects are reduced (the latter to essentially unity) in this reaction, compared to the aqueous acyl transfer from PNPA to oxygen nucleophiles such as the anion of hexafluoro-2-propanol. This pattern is consistent with protonation of the leaving group in the transition state, which is reasonable given the pH of the reaction. The magnitudes of these isotope effects are similar to previous observations of reduced phenolic ^{18}O and ^{15}N kinetic isotope effects for reactions in which protonation is concurrent with nitrophenol expulsion: the aqueous hydrolysis of *p*-nitrophenyl phosphate monoanion[9] and protein-tyrosine phosphatase catalyzed *p*-nitrophenyl phosphate hydrolysis.[17] These reactions have transition states with a large degree of bond cleavage to the leaving group and concurrent protonation, and thus the same is implied in the acid protease reaction. Since bond cleavage to the leaving group remains significant, and $^{18}k_{carbonyl}$, ^{D}k, and ^{13}k remain very similar to the other reactions, a concerted acyl transfer is implicated in this reaction as well.

Hyperconjugation. All four enzymes studied exhibited β-D kinetic isotope effects which are much less inverse than for similar nonenzymatic reactions in water. A possible explanation is that the oxyanion hole or other polarizing group is involved in an electrophilic interaction with the carbonyl oxygen. This hydrogen bonding will lead to increased hyperconjugation in the initial enzyme−substrate complex. While there will be less hyperconjugation in the subsequent transition state, the resulting degree of hyperconjugation may not be very different from that in the free substrate. This would result in a diminution of the β-deuterium isotope effects, which is what is observed in each of the enzymatic reactions, particularly in the case of carbonic anhydrase (Table 1).

The hyperconjugation effect is due to orbital overlap between the CH σ and carbonyl π^* orbitals. The Lewis acidity of the π^* orbital will be increased through hydrogen bonding or electrophilic interaction and hyperconjugation will be increased. Another way of viewing this is that the carbon lobes of the π^* orbital will increase in size as the π orbital is polarized toward the oxygen. If the transition state lies on the pathway to a stable

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tetrahedral intermediate, then by the Hammond postulate it should resemble that intermediate and have nearly sp^3 hybridization at the carbonyl carbon. Such a transition state would have minimal hyperconjugation due to the lack of a π orbital acceptor, and interactions of the carbonyl oxygen would have only a small effect on hyperconjugation. However, if the reaction is concerted, as the isotope effects suggest, the carbonyl group will have more π character in the transition state and electrostatic interaction with the carbonyl will increase hyperconjugation. Using this hypothesis, one may predict that the stepwise cleavage of less activated esters will show isotope effects more inverse than the ones seen here. Indeed, the deacylation of chymotrypsin acyl-enzyme has a β-D kinetic isotope effect of 0.94 ± 0.01.[18]

To estimate the magnitude of bond order changes and energetic contributions from the hyperconjugation interaction, gas-phase ab initio calculations were performed on acetaldehyde, protonated acetaldehyde, a complex of acetaldehyde hydrogen bonded to ammonium ion, acetaldehyde hydrate, and the hydroxide adduct of acetaldehyde (i.e., deprotonated acetaldehyde hydrate). The Natural Bonding Orbital method of Weinhold et al. uses ab initio electron density to find a set of bond orbitals which best describes the molecule. The program also computes perturbation energies for orbital interactions such as the donation of electrons from bonding to antibonding orbitals. The Natural Resonance Theory calculation assigns weights to various resonance forms of the molecule. These programs are uniquely suited to the modeling of hyperconjugation.

Perturbation analysis of natural bond orbital electron donor–acceptor interactions shows that the σ CH $\rightarrow$ π^* CO interaction is the hyperconjugative interaction that has the greatest increase upon polarization of the carbonyl group. Other less important delocalizations are the σ CH $\rightarrow$ σ^* CO and $\pi \rightarrow \sigma^*$ interactions. The two CH bond orbitals oriented anti to the carbonyl group interact twice as strongly with the carbonyl π^* orbital when acetaldehyde is protonated or complexed with ammonia (Table 2). Protonation of or hydrogen bonding to the carbonyl oxygen of acetaldehyde increases the perturbation energy of the σ CH to π^* CO interaction. The contour plot of the orbital overlap in Figure 5 shows greater overlap in the protonated acetaldehyde. Natural Resonance Theory analysis shows that the resonance form in which these CH bonds are broken increases in weight from 0.9% in the hemiacetal to 1.8% in acetaldehyde to 3.1% in the ammonia complex and 3.9% in the protonated species. Populations of the σ CH bonding orbital decrease and CO π^* orbitals increase as expected. Average bond order is decreased from 0.9939 in the hydrate, 0.9911 in acetaldehyde to 0.9867 in the ammonium ion complex and 0.9847 in the protonated form. Electrophilic interaction with the carbonyl group increases the p character of the carbon side of the CO hybrid bond orbitals from 67.7% for acetaldehyde to 71.8% for the protonated form; the increased Lewis acidity of the π orbital in the protonated and hydrogen bonded form contributes more hyperconjugation than is lost due to changes in bonding and geometry associated with this rehybridization.

If the isotope effect is linearly related to changes in the average C−H bond order, as suggested by Hogg et al.,[19] then an isotope effect may be estimated from the present bond order computations. Using 0.87 for the equilibrium isotope effect on hydration of acetaldehyde and the calculated average bond orders for acetaldehyde and its hydrate yields isotope effects

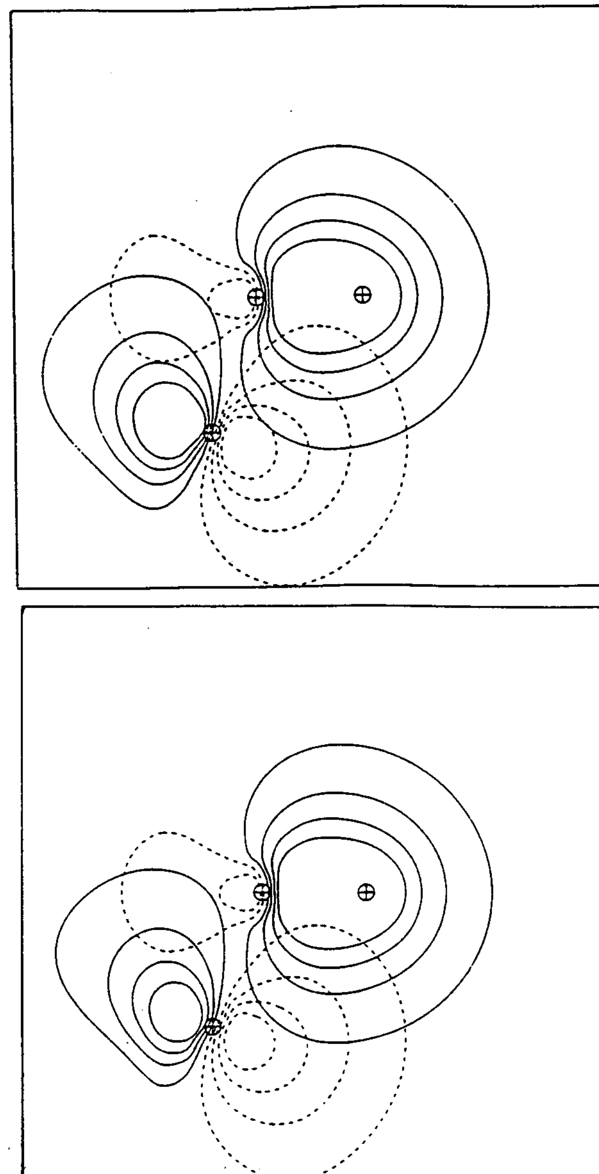

Figure 5. Contour plots showing overlap of CH σ and CO π^* prenatural bonding orbitals. The plane of the page is along an H−C−C axis. The top diagram was generated from the 6-31G* optimized acetaldehyde. The bottom diagram represents the 6-31G* optimized protonated acetaldehyde. The three atoms are one of the anti hyperconjugating hydrogens and the two carbon atoms. The oxygen atom is located out of the plane of the page as is the orientation of the π^* orbital.

of 1.22 and 1.32 per three deuteriums for hydrogen bonding to ammonium ion and for full protonation, respectively. The isotope effects on the enzymatic reactions may be thought of as being the product of the inverse isotope effect arising from nucleophilic attack (with associated loss of hyperconjugation) and the normal isotope effect (0.948) arising from hydrogen bonding to the carbonyl oxygen atom. Assuming that the former contribution is similar to the isotope effect for the attack of the anion of hexafluoro-2-propanol in solution, then in the chymotrypsin reaction the observed isotope effect of 0.982 is reduced from the solution value by a normal isotope effect of 1.036, which presumably arises from hydrogen bonding. This enzyme-induced reduction of the β-D kinetic isotope effect is on the order of 16 % of the isotope effect for ammonium ion complexation to acetaldehyde. It would not be unreasonable to suggest that two hydrogen bonds from the oxyanion hole of

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an enzyme could together contribute this fraction of the polarizing effect of complexation with ammonium ion in the gas phase. In the case of carbonic anhydrase, the enzyme-induced reduction in the β-D kinetic isotope effect amounts to about 22% of the isotope effect for ammonium ion complexation.

The calculated β-deuterium isotope effect of 1.32 for three deuterium atoms on protonation of acetaldehyde may be compared with the recently calculated isotope effect of 0.97 per deuterium for deprotonation of protonated acetone. This calculation was done with force constants generated from MP2/6-31G* optimized geometries and corresponds to an isotope effect of 1.19 for six deuteriums.[20] The natural bond orbitals are a linear combination of the two hybrid orbitals ($\pi^{*CO} = c_a p_c + c_b p_o$), and $100|c_a|^2$ is a useful measure of the polarization of the orbital and is an important predictor of the extent of hyperconjugation. This value increases from 69.06% in acetaldehyde, to 74.8% in the ammonium ion-acetaldehyde complex, and to 83.44% in fully protonated acetaldehyde.

Using deprotonation of the hydrate as a model of the anionic tetrahedral species demonstrates that the enzymatic contribution to ^{D}k cannot be explained by hydrogen bonding to an intermediate in which the carbonyl carbon is singly bonded to the ionized oxygen. Adding a proton to the oxygen of the tetrahedral intermediate model increases the average CH bond order from 0.9922 to 0.9936 and predicts that hydrogen bonding by an enzyme to such an intermediate would make ^{D}k more inverse than for a corresponding solution reaction. Our observation of a less inverse ^{D}k for the enzyme-catalyzed reactions relative to the nonenzymatic reactions supports the hypothesis of a concerted acyl transfer from PNPA in both nonenzymatic and enzyme-catalyzed reactions. The C−H bond order gained on protonation of the oxyanion hydrate of acetaldehyde may be due to shorter equilibrium CH and CC bond lengths in the neutral species which is at least partly associated with the loss of contribution from the species shown to the right in Figure 6, in which the oxyanion is bonded to either of two acetyl hydrogens and a formal negative charge is placed on the methyl carbon.

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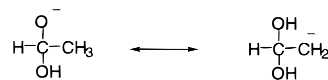

Figure 6. Representation of a minor tautomer of the deprotonated hydrate of acetaldehyde.

Although there appears to be hydrogen bonding or electrostatic interaction between the enzyme and the carbonyl oxygen, the $^{18}k_{carbonyl}$ values are not noticeably altered from the nonenzymatic phenolate reaction. The hydrogen-bonding interactions are presumably entirely electrostatic and do not result in a measurable ^{18}O isotope effect. Negligible ^{18}O effects resulting from electrostatic interactions have been seen previously for Mg^{2+} coordination to the oxygen of ATP.[21]

Conclusions

Enzyme-catalyzed PNPA hydrolysis proceeds by the same concerted mechanism as the uncatalyzed reaction. The transition state has some tetrahedral character, but maintains partial carbonyl π bonding. The decrease in magnitude of the β-deuterium isotope effect for enzyme-catalyzed reactions relative to similar nonenzymatic reactions appears to be due to polarization of the carbonyl group at the enzymatic active sites. Model calculations for hydrogen bonding to acetaldehyde demonstrate the nature and maximum magnitude of the enzyme-induced effect and show that the phenomenon of decreased β-deuterium isotope effects is unique to a transition state with significant carbonyl π bonding and may be unique to concerted enzymatic acyl transfers from esters.

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