Acid-Catalyzed Hydrolysis of 5-Enolpyruvylshikimate 3-Phosphate (EPSP) and Some Simple Models of Its Vinyl Ether Functional Group

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Abstract: Rates of hydrolysis of the vinyl ether functional groups of 5-enolpyruvylshikimate 3-phosphate (EPSP), α-methoxyacrylic acid, and methyl α-methoxyacrylate were measured in concentrated and dilute mineral acid solutions and carboxylic acid buffers, and rate profiles were constructed from the data so obtained. The results for methyl α-methoxyacrylate show a monotonic decrease in reaction rate with decreasing solvent acidity, culminating in a first-order dependence on $[H^+]$ in dilute acids; this and solvent isotope effects on this reaction indicate that this process occurs by the conventional mechanism for vinyl ether hydrolysis involving rate-determining proton transfer from hydronium ion to the substrate. The rate profiles for EPSP and α-methoxyacrylic acid are similar to that of methyl α-methoxyacrylate in concentrated acids, but they show uncatalyzed plateaus in the region $pC_{H^+} = 1-3$ that then give way to renewed acid catalysis beyond $pC_H = 4$. Solvent isotope effects suggest that these plateaus represent reaction via ionization of the carboxylic acid groups of these substrates followed by conventional hydrolysis of the vinyl ether groups of the carboxylate ions. Analysis of the data gives $pK_a = 3.77$ for EPSP and $pK_a = 3.46$ for α-methoxyacrylic acid and provides rate constants which show rate-retarding substituent effects on vinyl ether hydrolysis of 16 for α-CO_2^-, 20 000 for α-CO_2H, and 81 000 for α-CO_2Me.

The vinyl ether 5-enolpyruvylshikimate 3-phosphate (EPSP) (**1**) is the product of an unusual enzymatic reaction in the shikimic acid pathway catalyzed by EPSP synthase (EPSPS, EC 2.5.1.19).[1] This enzyme has been the subject of ongoing investigations because it functions as the biological target for glyphosate, the active ingredient in ROUNDUP herbicide,[2,3] whose discoverer was recently honored by the award of the Perkin Medal of the Society of Chemical Industry.[4] EPSPS transfers the carboxyvinyl group from phosphoenolpyruvate (PEP) regiospecifically to the 5-OH of shikimate 3-phosphate (S3P), forming EPSP (Scheme I). Such transfer reactions are relatively rare for PEP-utilizing enzymes, and a nonenzymatic analogy for either the forward or the reverse reaction in solution has yet to be demonstrated.

The mechanism of the EPSPS-catalyzed reaction proceeds through a single, kinetically competent, tetrahedral intermediate **2**, presumably via protonation of either PEP or EPSP at C-3 during catalysis. Evidence for this intermediate comes from rapid quench kinetics,[5] as well as isolation of the enzyme-bound intermediate[6] and its observation by ^{13}C NMR spectroscopy.[7] Reversible protonation of PEP and EPSP during catalysis is also indicated, inasmuch as EPSPS will catalyze deuterium exchange from the solvent into the terminal vinylic protons of PEP and EPSP in the presence of D_2O.[8-10] No comparable exchange is observed with either PEP or EPSP alone in solution in the absence of enzyme. EPSPS also catalyzes the hydrolysis of EPSP to S3P and pyruvate in a slow side reaction off the normal catalytic pathway.[5,7]

The acid-catalyzed hydrolysis of vinyl ethers is a prototypical rate-determining proton-transfer reaction whose detailed investigation has produced considerable insight into the proton-transfer process and has led to a well-defined reaction mechanism with no apparent exception.[11] None of the systems examined to date, however, have contained the complex functionality and ionizable substituents present in EPSP. In order to characterize the proton-transfer behavior of EPSP, we have examined the hydrolysis in aqueous solution of the vinyl ether group of EPSP as well as those of two simpler models, α-methoxyacrylic acid (**3**) and methyl α-methoxyacrylate (**4**). The results we have obtained add further insight into the mode of EPSP recognition by EPSPS and the nature of the catalytic events occurring at the enzyme active site.

3 **4**

Experimental Section

Materials. EPSP was prepared as described previously.[5] α-Methoxyacrylic acid was obtained by base-catalyzed hydrolysis of its methyl ester,[12] which was synthesized by treating methyl 2,3-dibromopropionate with methoxide ion.[13] The acid was purified by recrystallization from pentane or hexane, and the ester was purified by gas chromatography. The physical properties of these substances agreed with literature values, and their NMR and IR spectra were consistent with their structures. All other materials were the best available commercial grades. Solutions were made from distilled H_2O or D_2O (Merck Sharp and Dohme, 99.9 atom % D) as received.

pK_a Determination. The pK_a of α-methoxyacrylic acid was determined potentiometrically by measuring the pH of partly neutralized aqueous

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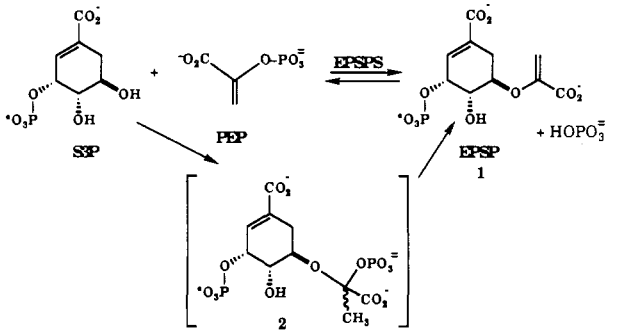
Scheme I



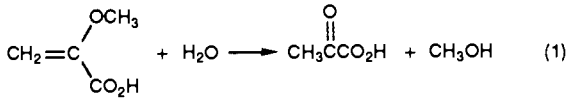
solutions of this acid at several different concentration ratios, R (= [HA]/[A$^-$]). The ionic strength of these solutions was maintained at 0.10 M by adding $NaClO_4$ as required, and measurements were made with a Beckman Model 1019 Research pH meter. The results were used to calculate pK_a values according to the relationship pK_a = pH + log R − log f_{A^-}, in which f_{A^-} is the activity coefficient of the α-methoxyacrylate ion; this was taken to have the value recommended by Bates for acetate ion, f_{A^-} = 0.775.[14]

Kinetics. Rates of hydrolysis of the vinyl ether groups of the substrates were determined spectroscopically by monitoring the decrease in carbon–carbon double bond absorbance of these substances at λ = 220–230 nm. Measurements were made with Cary Model 118 and 2200 spectrometers whose cell compartments were thermostated at 25.0 ± 0.05 °C. Substrate concentrations in the reaction mixtures were ca. 10^{-4} M.

The hydrolysis reactions were generally followed to completion. The data so obtained fit the first-order rate law well, and observed first-order rate constants were calculated by least-squares fit to an exponential function. For some of the slower runs, an initial-rate method was also employed. This involved measuring the linear decrease in absorbance over the first few percent reaction, using an offset scale of the spectrometer, and then converting the zero-order rate so obtained into a first-order rate constant by dividing by the initial absorbance reading corrected for the absorbance of the reaction solution without substrate. This initial-rate method requires the reaction products to have no absorbance at the wavelength employed, a condition that is fulfilled for α-methoxyacrylic acid and methyl α-methoxyacrylate, but not for EPSP because the product of its hydrolysis, S3P (Scheme I), still contains a strongly absorbing carbon–carbon double bond; the method was consequently not used to determine rate constants for EPSP.

Results

Product Studies. The course of the hydrolysis of α-methoxyacrylic acid in 0.10 M DCl/D_2O was studied using ^{1}H NMR spectroscopy to monitor the changes that occurred. The initial spectra consisted of doublets at δ (ppm) 5.25 and 4.70 (J = 3.2 Hz, 1 H each) that could be attributed to the vinyl hydrogens of the substrate and another signal at δ (ppm) 3.48 (s, 3 H) due to its methoxy group. These resonances gradually diminished in intensity and were replaced by a new singlet at δ (ppm) 3.15 and multiplets at δ (ppm) 2.24 and 1.37; these were identified, by addition of authentic samples, as originating from methanol and the methyl groups of unhydrated and hydrated pyruvic acid, respectively. The multiplet at δ (ppm) 2.24 was a clean triplet, J = 2.3 Hz, with no evidence of further fine structure, as expected for the protons in a CH_2D group split by the single deuteron; the multiplet at δ (ppm) 1.37 was unresolved. These observations indicate that the acid-catalyzed hydrolysis of α-methoxyacrylic acid occurs cleanly, as expected according to eq 1, and that only one hydrogen enters the product from the solvent in the course of this reaction; the hydrogen transfer from catalyst to substrate that occurs during this reaction is thus not reversible.



(1)

Less detailed product studies were conducted on the hydrolyses of methyl α-methoxyacrylate and EPSP. These showed that these reactions were also straightforward, giving only the products expected from hydrolysis of their vinyl ether functional groups.

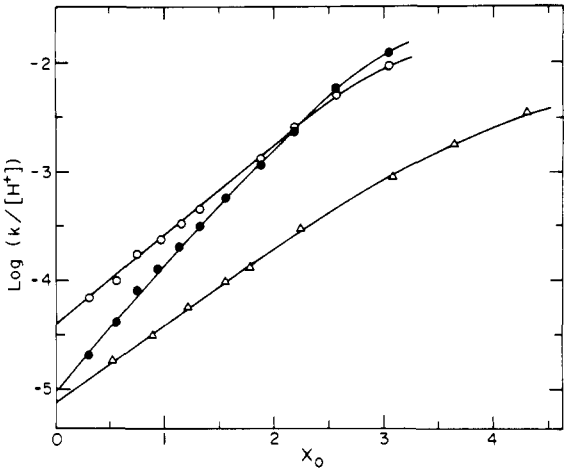


Figure 1. Acidity function correlations for hydrolysis of the vinyl ether groups of α-methoxyacrylic acid (○), methyl α-methoxyacrylate (●), and EPSP (△) in concentrated aqueous perchloric acid at 25 °C.

pK_a of α-Methoxyacrylic Acid. The acidity constant of α-methoxyacrylic acid was determined by measuring the pH of solutions of this acid and its conjugate base whose concentration ratios, R (= [HA]/[A$^-$]), varied from 0.5 to 2.5. Four solutions were used, and the pH of each solution was measured five times. The results gave pK_a = 3.394 ± 0.005; this statistical uncertainty, however, does not take into account possible systematic errors, and a plausible estimate of those leads to pK_a = 3.39 ± 0.02 as the most probable value of this quantity. This result is consistent with the acidity constant of α-methoxyacrylic acid obtained from analysis of its hydrolysis kinetics (vide infra), but it differs considerably from a value, pK_a = 3.80, that can be estimated using a σ–ρ correlation for α-substituted acrylic acids;[15] the reason for this difference is not apparent.

Kinetics, Concentrated Acids. Rates of hydrolysis of the vinyl ether functional groups of the presently examined substrates were measured in concentrated aqueous perchloric acid solutions over the concentration range 10–50 wt % acid (1–8 M) for α-methoxyacrylic acid and its methyl ester and 20–60 wt % acid (2–9 M) for EPSP. The kinetic data so obtained are summarized in Tables S1–S3 of the supplementary material.[16]

All three reactions are strongly catalyzed by acid, and observed first-order rate constants increase even more rapidly than in direct proportion to acid concentration. Such behavior is not uncommon for reactions in concentrated acid solution, and the situation is usually handled by using an acidity function to correlate the data. The X_0 function[17] appears to be the best scale currently available for this purpose.[18]

Correlations using this acidity function are normally made by fitting the data to an expression of the form of eq 2, in which k_{H^+} is the bimolecular catalytic coefficient that applies in dilute acid solution where X_0 = 0 and m^* is a slope parameter. This

$$\log (k_{obsd}/C_{H^+}) = \log k_{H^+} + m^* X_0 \qquad (2)$$

treatment implies that log (k_{obsd}/C_{H^+}) is a linear function of X_0 but, as Figure 1 shows, that is not the case for any of the substrates examined here; although the relationship is reasonably linear at low acidities, curvature develops in the more concentrated solutions. Such behavior has been observed before in the hydrolysis of vinyl ethers,[19] and it has been attributed to partial conversion

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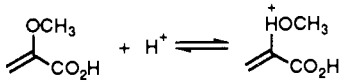
Table I. Summary of Parameters Governing the Equilibrium Oxygen Protonation and Rate of Vinyl Ether Hydrolysis of the Presently Examined Substrates in Aqueous Solution at 25 °C

parameter	α-methoxyacrylic acid	methyl α-methoxyacrylate	EPSP
pK_{SH^+}		−3.37 ± 0.30	−2.88 ± 0.21
m		0.89 ± 0.09	0.52 ± 0.04
$k_{H^+}/10^{-5}$ M^{-1} s^{-1}	3.81 ± 0.06	0.944 ± 0.028	0.743 ± 0.022
m^*	0.82 ± 0.01	1.15 ± 0.01	0.73 ± 0.01
$k'_{H^+}/10^{-2}$ M^{-1} s^{-1} [a]	4.75 ± 0.39		3.94 ± 0.52
pQ_a [a]	3.27 ± 0.04		3.58 ± 0.06

[a] At an ionic strength of 0.10 M.

of the substrate into a less reactive form through rapid equilibrium protonation on the ether oxygen, as illustrated for α-methoxyacrylic acid in eq 3.



(3)

This situation requires a modified form of eq 2, shown as eq 4, in which the additional parameters K_{SH^+} and m are the acidity constant of the oxygen-protonated substrate and the slope governing this equilibrium protonation, respectively. A least-squares

$$\log (k_{obsd}/[H^+]) = \log k_{H^+} + m^* X_0 + \log \left(\frac{K_{SH^+}}{K_{SH^+} + [H^+]10^{mX_0}} \right) \quad (4)$$

fit of the data to this expression gave the results listed in the first four rows of Table I. There is insufficient curvature in the data for α-methoxyacrylic acid to give a statistically significant value of K_{SH^+} for this substrate, but this parameter could be defined for the other two substances examined; the values obtained, $pK_{SH^+} = -3.4$ for methyl α-methoxyacrylate and $pK_{SH^+} = -2.9$ for EPSP, are similar to those determined for other vinyl ethers[19] and are typical of pK_a's for conjugate acids of simple aliphatic ethers.[20]

One of the presently examined substrates, methyl α-methoxyacrylate, contains another functional group, its carboxylic acid ester substituent, which is also susceptible to hydrolysis in aqueous acid solutions. The data obtained, however, indicated that hydrolysis of this group did not interfere with the vinyl ether reaction studied. If ester hydrolysis had occurred more rapidly than vinyl ether hydrolysis, then the substrate would have been converted into α-methoxyacrylic acid, and the kinetic parameters obtained would have been the same as those determined for that substance; the data listed in Table I show that this is not so. If, on the other hand, the two reactions had occurred at comparable rates, deviations from first-order behavior would have resulted, but good compliance with the first-order rate law was actually found. This leaves ester hydrolysis as the significantly slower reaction, which is consistent with the fact that the specific rate of acid-catalyzed hydrolysis of methyl acrylate[21] is only half as great as the rate constant determined here and the further expectation that the bulk of the α-substituent in the present substrate would provide an additional retardation of its ester hydrolysis reaction.

Kinetics, Dilute Acids. Rates of hydrolysis of the vinyl ether functional groups of two of the presently studied substrates, α-methoxyacrylic acid and EPSP, were also measured in dilute aqueous perchloric acid solutions (0.0005–0.10 M) and aqueous buffer solutions of methoxyacetic and acetic acids. These data are summarized in Tables S4–S7 given in the supplementary material.[16]

The measurements in the buffers were performed in series of solutions of constant buffer ratio and constant ionic strength (0.10 M) but varying buffer concentration. This served to hold hydrogen ion concentrations constant along a given solution series; buffer catalysis could then be assessed by constructing buffer dilution plots. Moderately strong buffer catalysis was found, and a comparison of the results for different buffer ratios showed this to be of the general acid type, as expected on the basis of the conventional mechanism for vinyl ether hydrolysis.[11,22] The zero-buffer-concentration intercepts of these buffer dilution plots were used to construct the dilute acid regions of the rate profiles shown in Figure 2.[23]

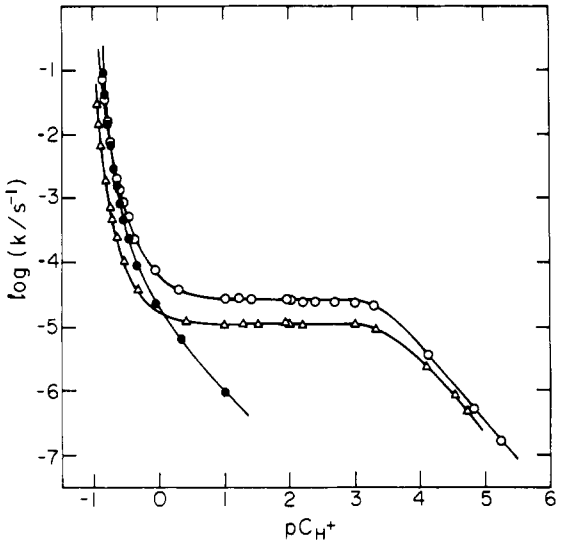


Figure 2. Rate profiles for the hydrolysis of the vinyl ether groups of α-methoxyacrylic acid (○), methyl α-methoxyacrylate (●), and EPSP (△) in aqueous solution at 25 °C.

Table II. Solvent Isotope Effects on Vinyl Ether Hydrolysis of the Presently Examined Substrates in Aqueous Solution at 25 °C

	k_H/k_D		
$[L^+]/M$ [a]	α-methoxyacrylic acid	methyl α-methoxyacrylate	EPSP
0.49	4.59	1.64	
0.39			4.82
0.099	5.84	1.59	
0.011	7.24		5.71

[a] $L^+ = H^+$ or D^+.

Solvent Isotope Effects. Rates of vinyl ether hydrolysis for the presently examined substrates were measured in H_2O and D_2O solutions of perchloric or hydrochloric acid at several concentrations over the range 0.5–0.01 M. Pairs of determinations were made at the same acid concentration in the two solvents, and isotope effects were calculated by dividing rate constants determined in H_2O by those determined in D_2O. The rate data are summarized in Table S8 of the supplementary material,[16] and the isotope effects are listed in Table II.

Discussion

Reaction Mechanism. The behavior of all three of the presently examined substrates in concentrated acid solution is typical of simple vinyl ether hydrolysis reactions. The rates of hydrolysis of all three substrates increase with acidity even more rapidly than in direct proportion to acid concentration, as is commonly found for vinyl ether hydrolysis.[19,24] The present rate data, moreover, correlate well with the X_0 acidity function,[17] giving slopes not greatly different from unity, except at very high acidities where characteristic deviations attributable to ether oxygen protonation occur; this again is quite similar to behavior shown by other simple

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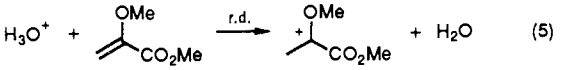
(21) Salmi, E. J. *Ber.* **1939**, *72B*, 1767–1777.
(22) Kresge, A. J. *Acc. Chem. Res.* **1987**, *20*, 364–370.
(23) Hydrogen ion concentrations of the buffer solutions were obtained by calculation using literature pK_a's and activity coefficients recommended by Bates.[14]
(24) Chiang, Y.; Kresge, A. J.; Young, C. I. *Can. J. Chem.* **1978**, *56*, 461–464.

vinyl ether hydrolysis reactions.[19]

For methyl α-methoxyacrylate (but not for the other two substrates; vide infra), this concentrated acid behavior develops into a first-order dependence of rate on hydronium ion concentration in dilute acids. This is illustrated in Figure 2, where the line drawn is based upon parameters determined from rates of hydrolysis of this substrate in concentrated acids. The good correspondence of this line with the experimental data in dilute acid shows that the latter derive smoothly from the behavior in concentrated acids, as expected on the basis of the conventional mechanism for vinyl ether hydrolysis.[11,22]

Solvent isotope effects on the hydrolysis of methyl α-methoxyacrylate also point to the operation of the conventional mechanism for this substrate. The data of Table II show that these isotope effects do not change with changes in acidity, as expected, and that they are quite small, as anticipated for a vinyl ether of this low reactivity. Their magnitude, $k_H/k_D = 1.6$, is in fact consistent with $k_H/k_D = 1.2$ predicted from a correlation of isotope effects with free energies of activation for a large group of vinyl ether hydrolyses known to occur by the conventional mechanism.[25] It would seem safe to conclude, therefore, that methyl α-methoxyacrylate undergoes hydrolysis by the conventional mechanism as well, namely, by rate-determining proton transfer from the hydronium ion catalyst to the β-carbon atom of the substrate (eq 5), followed by rapid hydration of the cation so formed and decomposition of the subsequent hemiketal intermediate (eq 6).



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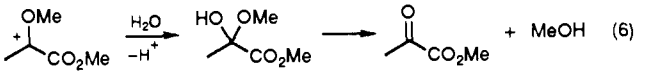
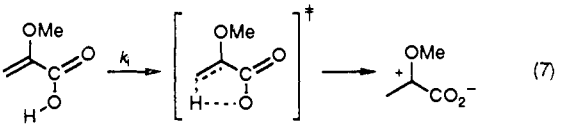


(6)

The other two substrates give quite different behavior: as Figure 2 shows, their rates of reaction are independent of acidity over the range $pC_{H^+} = 1-3$. Acid catalysis does reappear in more dilute solutions, but rates of reaction are now considerably greater than expected on the basis of data obtained in concentrated acids.

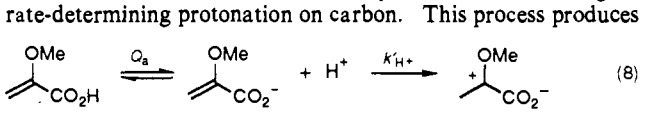
These two substrates, of course, unlike methyl α-methoxyacrylate, have carboxylic acid groups, and the fact that the change from no catalysis to renewed acid catalysis occurs at pC_{H^+} values where these carboxylic acid groups are expected to ionize implicates these groups in this different behavior. Two explanations consistent with this implication may be advanced. The first is an intramolecular reaction in which the carboxylic acid group of the substrate is functioning as a general acid catalyst in the rate-determining proton-transfer step; this is shown as a direct reaction in eq 7, but the proton transfer could also occur via an intervening solvent water molecule. Such a process would benefit



(7)

from the well-known superiority of intramolecular over intermolecular reactions,[26] and it would give a rate dependent only on substrate concentration and independent of acidity, as long as the substrate remained in its un-ionized carboxylic acid form.

The second explanation, shown as eq 8, involves ionization of the carboxylic acid group to give a more reactive form of the substrate; this form, i.e., the carboxylate ion, then undergoes rate-determining protonation on carbon. This process produces



(8)

a proton in the first preequilibrium step and then uses it up in the second, rate-determining step; the overall reaction is therefore independent of proton concentration, and it will remain so as long as the substrate stays in its un-ionized carboxylic acid form.

The rate laws for these two reaction schemes have the same mathematical form (eq 9). The interpretation of the rate constant, k, in this rate law on a molecular basis, however, is different for the two cases. For the mechanism of eq 7, it is equal to the

$$k_{obsd} = \frac{k[H]^+}{[H^+] + Q_a} \qquad (9)$$

specific rate of the intramolecular process, $k = k_i$, whereas for the mechanism of eq 8 it is equal to the hydronium ion catalytic coefficient for reaction of the carboxylate form of the substrate, k'_{H^+}, multiplied by the concentration quotient for ionization of its carboxylic acid group at the ionic strength of the rate measurements, Q_a, $k = k'_{H^+}Q_a$. In both cases, observed rate constants will be independent of acidity when $[H^+] \gg Q_a$ and will decrease with decreasing acidity when $[H^+] \ll Q_a$, as observed.

Because the rate laws for these two mechanisms have the same mathematical form, the mechanisms cannot be distinguished on the basis of kinetic data alone. Kinetic isotope effects, however, might be diagnostic inasmuch as they reflect transition-state structure, which, of course, is different for the two reaction schemes. The isotope effect on the intramolecular mechanism by direct proton transfer as shown in eq 7 is expected to be small because the transition state here will be bent, i.e., the angle formed by the partial bonds to the hydrogen in transit will certainly be much less than 180°, and it may be as small as 90°. Calculations[27] have shown that an isotope effect of $k_H/k_D = 7.9$ when this angle is 180° is reduced to $k_H/k_D = 3.0$ for an angle of 120° and to $k_H/k_D = 1.7$ for an angle of 90°. Inclusion of a water molecule in this transition state would allow the angle to open up to 180°. The isotope effect, however, would still be smaller than the values, $k_H/k_D = 5-7$, observed for the hydrolysis of other vinyl ethers catalyzed by carboxylic acids[28] inasmuch as the present reactions are 4–7 orders of magnitude slower than the other hydrolyses; such large rate reductions can be expected to reduce the symmetry of the transition state significantly and thus considerably diminish the isotope effect.[25,29] The isotope effect on reaction by the pre-ionization mechanism of eq 8, on the other hand, will be large because it consists of a kinetic isotope effect on the rate-determining step multiplied by an equilibrium isotope effect on the prior acid ionization.

Table II shows that the isotope effects on the hydrolysis of α-methoxyacrylic acid and EPSP, unlike the isotope effects on the hydrolysis of methyl α-methoxyacrylate, increase as the acid concentration drops from 0.5 to 0.01 M. This is to be expected on the basis of both mechanisms and is not diagnostic. At the higher acid concentrations, some reaction still occurs through the mechanism that applies in concentrated acids, and the low isotope effect on this process, e.g., $k_H/k_D = 1.6$ observed for methyl α-methoxyacrylate, reduces the observed effect. At acid concentrations on the order of 0.01 M, however, essentially all of the reaction occurs by the mechanism that gives rise to the plateaus in the rate profiles for these systems, and it is the isotope effects at this acidity that are characteristic of this mechanism.

These isotope effects are large. The values $k_H/k_D = 7.2$ and 5.7 are certainly larger than expected for reaction by direct intramolecular proton transfer through a bent transition state (eq 7) and probably also larger than expected for proton transfer via an intervening water molecule. They are, however, consistent with the pre-ionization mechanism of eq 8. For this reaction scheme, $(k_H/k_D)_{obsd} = (k'_{H^+}/k'_{D^+})(Q_{a,H}/Q_{a,D})$, and the use of estimates of the kinetic isotope effect, k'_{H^+}/k'_{D^+}, predicted from the cor-

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relation of isotope effects on other vinyl ether hydrolysis reactions,[25] namely, k'_{H^+}/k'_{D^+} = 2.4 for α-methoxyacrylic acid and k'_{H^+}/k'_{D^+} = 2.3 for EPSP, leads to the equilibrium isotope effects $Q_{a,H}/Q_{a,D}$ = 3.1 for the former and $Q_{a,H}/Q_{a,D}$ = 2.5 for the latter. These are eminently reasonable values for isotope effects on the equilibrium ionization of carboxylic acids of this acid strength; for example, $K_{a,H}/K_{a,D}$ = 2.6 for iodoacetic acid,[30] and $K_{a,H}/K_{a,D}$ = 2.8 for formic acid.[30-32]

Solvent isotope effects thus suggest that the hydrolysis of α-methoxyacrylic acid and EPSP in the plateau regions of their rate profiles occurs by the pre-ionization mechanism of eq 8 and not by intramolecular proton transfer. This assignment is supported by the general ineffectiveness of intramolecular catalysis in vinyl ether hydrolysis: intramolecular catalysis of this reaction has been difficult to find and, in the few cases where it has been established, it has proven to be quite weak.[33]

The version of the rate law of eq 9 that applies to the pre-ionization mechanisms of eq 8 is shown as eq 10. A least-squares fit of the kinetic data to this expression produced the values of k'_{H^+} and Q_a listed in Table I. The parameter Q_a is a concentration

$$k_{obsd} = \frac{k'_{H^+}Q_a[H^+]}{[H^+] + Q_a} \qquad (10)$$

quotient applicable at an ionic strength of 0.10 M; it may be converted to a zero-ionic-strength acidity constant, K_a, by including activity coefficients, f, for the ionic species involved in the acid ionization reaction. When this is done using f = 0.83 for the hydronium ion and f = 0.775 for the carboxylate ions,[14] pK_a = 3.46 ± 0.04 is obtained for α-methoxyacrylic acid and pK_a = 3.77 ± 0.06 for EPSP. The former agrees well with pK_a = 3.39 ± 0.02 determined directly by pH measurements (vide supra).

Reactivity. The results obtained here show that a carboxylate group situated in the α-position has only a mild retarding effect on the rate of vinyl ether hydrolysis; comparison of k'_{H^+} = 0.0475 M^{-1} s^{-1} for α-methoxyacrylic acid reacting in its ionized carboxylate form with k_{H^+} = 0.76 M^{-1} s^{-1} for the unsubstituted parent compound, methyl vinyl ether,[25] gives a 16-fold decrease in reactivity. Carboxylic acid and methyl carboxylate ester groups in the same position, on the other hand, have much stronger rate-retarding effects; comparisons similar to that made above for the carboxylate group give a factor of 20 000 for α-CO_2H, 102 000 for EPSP, and 81 000 for α-CO_2Me. These rate retardations are consistent with the destabilizing effects these groups are expected to have on the positive charge being generated on the substrate in the rate-determining step of the reactions, and the additional retardation shown by EPSP over that for α-methoxyacrylic acid may be understood in terms of an additional destabilization of this charge by the electron-withdrawing effect of the hydroxyl group at the 4-position of the shikimic acid portion of this substance.

It is interesting that these differences in reactivity, as well as the rate constants themselves, are consistent with predictions made from a correlation of specific rates of protonation by the hydronium ion of a large number of alkenes spanning 15 orders of magnitude in reactivity.[34] A comparison of predicted and observed values is provided in Table III. It may be seen that the average difference between prediction and observation, expressed in terms of differences in free energies of activation, is just 1.0 kcal mol^{-1}; this is probably better than the accuracy of the correlation. This good correspondence implies that the presently examined substrates

Table III. Comparison of Predicted and Observed Rates of Vinyl Ether Hydrolysis

substrate	k_{H^+}/M^{-1} s^{-1} predicted[a]	k_{H^+}/M^{-1} s^{-1} observed[b]	$\delta\Delta G^{\ddagger}$/ kcal mol^{-1} [c]
	2.3×10^{-1}	7.6×10^{-1} (1.00)	0.70
	4.1×10^{-1}	4.8×10^{-2} (1/16)	−1.27
	7.2×10^{-6}	3.8×10^{-5} (1/20 000)	0.98
	1.4×10^{-6}	9.4×10^{-6} (1/81 000)	1.15

[a] From log (k_{H^+}/M^{-1} s^{-1}) = −8.96 − 10.7$\sum\sigma^+$ (ref 34); σ^+ values taken from Leffler, J. E.; Grunwald, E. *Rates and Equilibria of Organic Reactions*; John Wiley & Sons: New York, 1963; p 204. [b] Relative values given in parentheses. [c] Predicted − observed.

are also reacting through protonation by the hydronium ion and not by intramolecular proton transfer from carboxylic acid groups situated in the substrates.

Comparison with Enzymatic Reaction. In addition to catalyzing the synthesis of EPSP from S3P and PEP (Scheme I), EPSP synthase also promotes the hydrolysis of EPSP to S3P and pyruvate. Despite the fact that this is only a minor function of this enzyme, the rate of the enzymatic reaction, $k = 4.7 \times 10^{-4}$ s^{-1} at 20 °C,[5,7] is nevertheless greater by 5 orders of magnitude than the nonenzymatic hydrolysis of EPSP would be expected to be at physiological pH on the basis of the rate constant, k'_{H^+}, measured here: $k'_{H^+}[H^+] = (3.9 \times 10^{-2})(1 \times 10^{-7}) = 3.9 \times 10^{-9}$ s^{-1}.

Conclusions. The results we have obtained illustrate the value of using the methods of physical organic chemistry to characterize enzymatic reactions. We have shown that the nonenzymatic hydrolysis of EPSP in aqueous solution occurs by the well-established mechanism for vinyl ether hydrolysis involving rate-determining proton transfer from catalyzing acid to substrate and, by measuring the rate of this reaction, we have provided a base against which an acceleration of 10^5 for catalysis of this reaction by EPSP synthase may be estimated. This is especially interesting in that EPSP hydrolysis is only a minor function of this enzyme and is therefore presumably not the goal toward which it evolved. We have also shown that EPSP is hydrolyzed more rapidly when its carboxylic acid group is in the carboxylate form than when this group is not ionized, which suggests that EPSP is recognized at the enzyme active site so as to maintain the ionized form of its carboxylate group during catalysis. This has an interesting bearing on the greater affinity of the enzyme for EPSP than for the hydrolysis product S3P:[35] whatever salt-bridge interaction is responsible for this differential affinity, it most likely occurs without formal proton transfer to EPSP's carboxylate functionality. A more detailed characterization of EPSP recognition by the enzyme is the subject of an ongoing study.

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Supplementary Material Available: Tables S1–S8 of rate data (9 pages). Ordering information is given on any current masthead page.

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