calculations show trends similar to those found here for **1**, the calculations tend to overestimate C–C bond lengths along the unsaturated chain. The most significant difference occurs with the C(13)–C(14) bond, for which the calculated distances are ca. 0.04 Å larger than those measured here.

In conclusion, **1** should serve as a good model for the chromophore of BR and provide detailed information on the structure of this important natural chromophore.

Supplementary Material Available: Tables containing atomic coordinates, bond lengths and angles, selected torsion angles, anisotropic Gaussian displacement parameters, hydrogen atom coordinates, and least-squares planes and a figure showing the stereoview of the molecular packing in the unit cell for the title compound (7 pages); listing of observed and calculated structure factor amplitudes of all reflections for the title compound (13 pages). Ordering information is given on any current masthead page.

Kinetic Isotope Effect Investigation of Enzyme Mechanism in Organic Solvents

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Enzymatic catalysis in anhydrous organic solvents (instead of water) has revealed exciting mechanistic phenomena[1] and led to new synthetic methodologies.[2] Nevertheless, our mechanistic understanding of enzyme action in such media, required to take a full advantage of these novel opportunities, is still in its infancy. Although recent studies of enzyme structure by solid-state NMR,[3] and mechanism by means of linear free energy correlations,[4] suggest profound similarities of those in water and in anhydrous solvents, no single method is sufficiently penetrating and only their repertoire can be truly conclusive. To this end, in the present work we have used a kinetic isotope effect approach to compare the mechanistic behavior of enzymes in various organic solvents.

Isotope effects on enzyme action have proven to be an insightful diagnostic tool.[5] Herein, we have initially applied it to a model reaction between vinyl butyrate and 1-butanol (BuOH or BuOD) catalyzed by subtilisin Carlsberg (protease from *Bacillus licheniformis*) in anhydrous acetonitrile. Such enzymatic transesterifications follow the compulsory order mechanism without ternary complexes:[6]

$$\text{E} + \text{ester} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{E·ester} \xrightarrow{k_2} \text{acyl-E} + \underset{\text{P}_1}{\text{alcohol}} \underset{k_{-1'}}{\overset{k_{1'}}{\rightleftharpoons}}$$

$$\text{acyl-E·alcohol} \xrightarrow{k_3} \text{E} + \text{P}_2 \quad (1)$$

where E is the enzyme, ester and alcohol are vinyl butyrate and butanol, respectively, and P_1 and P_2 are the reaction products. By measuring the dependence of the initial rate of the subtilisin-catalyzed transesterification on the concentration of the alcohol at a fixed ester concentration, we have determined the values of V and $K_m^{alcohol}$ for BuOH and BuOD. As one can see in the first line of Table I, both the maximal velocity and the Michaelis constant for BuOH are 3–4-fold greater than those for BuOD. Thus enzymatic reaction 1 in acetonitrile exhibits a pronounced deuterium kinetic isotope effect.

Table I. Primary Deuterium Kinetic Isotope Effects in the Enzymatic Transesterification Reaction between Vinyl Butyrate and 1-Butanol (BuOH or BuOD) in Various Anhydrous Organic Solvents[a]

enzyme	solvent	V^H/V^D	${}^HK_m^{alcohol}/{}^DK_m^{alcohol}$
subtilisin	acetonitrile	3.7 ± 0.7[b]	3.2 ± 0.7[c]
subtilisin	tetrahydrofuran	4.6 ± 0.8[b]	3.8 ± 0.6[c]
subtilisin	ethyl acetate[d]	4.6 ± 0.7[b]	3.1 ± 0.4[c]
lipase	acetonitrile	1.9 ± 0.2[b]	1.8 ± 0.2[c]
lipase	cyclohexane	1.5 ± 0.1[b]	1.9 ± 0.2[c]

[a] For both subtilisin- and lipase-catalyzed transesterifications, the ester concentration was 200 mM and the alcohol concentrations were varied in the range from 2 to 400 mM; the enzyme concentration was 1 mg/mL. Reaction mixtures (1 mL) were shaken at 300 rpm and 30 °C; periodically, 0.5-μL aliquots were withdrawn and analyzed by gas chromatography.[6] Organic solvents were dried prior to use to bring their water content below about 0.01% (for procedures, see ref 6). Subtilisin (Sigma Chemical Co., type VIII) was prepared by dissolving 5 mg/mL enzyme in 20 mM aqueous potassium phosphate buffer (pH 7.8), followed by lyophilization. Lipoprotein lipase from *Pseudomonas fluorescens* (Amano International Enzyme Co., type P 30) was merely extensively dried under vacuum prior to use. All reactions were initiated by the addition of the solid enzyme to the substrate solutions, followed by a 10-s ultrasonication of the resultant suspensions to homogenize them. The values of V and K_m were determined from the kinetic data by using a nonlinear regression analysis computer program (R. J. Leatherbarrow, "Enzfitter", Elsevier-BIOSOFT). Sample standard deviations were calculated on the basis of the average of at least three independent experiments. Error limits of the isotope effects were estimated by standard statistical techniques (Bevington, P. R. *Data Reduction and Error Analysis for the Physical Sciences*; McGraw-Hill: New York, 1969; pp 61, 113, 117). [b] The mean individual values for V^H and V^D were (in mM/min, from top to bottom) 0.081 and 0.022, 0.43 and 0.093, 0.55 and 0.12, 0.051 and 0.027, and 0.83 and 0.56, respectively. [c] The mean individual values for ${}^HK_m$ and ${}^DK_m$ were (in mM, from top to bottom) 200 and 63, 300 and 80, 470 and 150, 42 and 23, and 13 and 7, respectively. [d] No appreciable subtilisin-catalyzed reaction was observed between 1-butanol and ethyl acetate in the latter (i.e., in the absence of vinyl butyrate).

Analogous experiments have been carried out in two other, unrelated organic solvents. As can be seen in Table I, in anhydrous tetrahydrofuran and ethyl acetate, as in acetonitrile, subtilisin catalysis displays very similar deuterium kinetic isotope effects (whether for V or $K_m^{alcohol}$, as expected from the analysis of kinetic expressions for reaction 1[7]). It is worth mentioning that their magnitude is in the same range as those for hydrolysis reactions catalyzed by proteases and other hydrolases in water.[8]

In the simplest case,[7] the kinetic isotope effect observed (both V^H/V^D and ${}^HK_m^{alcohol}/{}^DK_m^{alcohol}$) equals k_3^H/k_3^D, i.e., corresponds to the reaction of acyl-subtilisin with the nucleophile (1-butanol). This deacylation process consists of at least four distinct steps:

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(7) See supplementary material. It should be pointed out that we were concerned about the possibility of D/H exchange among BuOD, adventitious water, and the enzyme, but ruled it out for the following reasons. In all cases, we observed perfectly linear initial rates (i.e., product concentration vs time dependences) for BuOD (as well as BuOH) as the nucleophile. Thus the putative D/H exchange is either almost instantaneous or does not occur to any significant extent (otherwise, the enzymatic transesterification would have accelerated with time for BuOD). The former possibility, however, is inconsistent with a large kinetic isotope effect observed for both V and K_m in different solvents. In addition, simple calculations (taking into account the water content in our system) indicate that the aforementioned D/H exchange, if occurring, may have significantly affected the BuOD concentration at 2–20 mM but not at 100–400 mM. Consequently, such an exchange would have resulted in deviations from linear Michaelis–Menten dependences which, in fact, were not observed.
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binding of the nucleophile to the acyl-enzyme, formation and subsequent decomposition of the tetrahedral intermediate, and product release.[9] The very fact that a substantial isotope effect is observed in subtilisin-catalyzed transesterification I indicates that the second step, namely, the abstraction of the proton from the hydroxyl group of the nucleophile by the catalytic triad's histidine,[10] is rate-limiting. Furthermore, since the magnitude of the primary deuterium kinetic isotope effect is related to the degree of proton transfer in the transition state,[11] the fact that this parameter is the same within experimental error for the subtilisin catalysis in different organic solvents (Table I) suggests a marked independence of the transition-state structure for the deacylation process on the nature of the reaction medium.

In order to test the generality of our findings with subtilisin, we have applied the deuterium kinetic isotope effect approach to transesterification I catalyzed by the lipase from *Pseudomonas fluorescens* in acetonitrile and in cyclohexane. As can be seen in the last two lines of Table I, for this (nonproteolytic) hydrolase, the magnitude of the isotope effect is nearly the same in both solvents, as is the case for the protease subtilisin. Therefore, combined with the results of Hammett analysis of the acylation of subtilisin,[4] the deacylation data obtained in this study support the notion of great similarities in the mechanism of enzymatic catalysis in different anhydrous solvents, whether the latter are miscible or immiscible with water and regardless of their chemical structure and physicochemical properties.

Supplementary Material Available: Analysis of kinetic expressions for reaction I and their implications (3 pages). Ordering information is given on any current masthead page.

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Highly Reduced Carbene Complexes: Formation of an Alkoxymalonate by Coupling of Carbon Dioxide with the Nucleophilic Carbene in $[Cr(CO)_4\{C(OMe)Ph\}]^{2-}$

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Fischer carbene complexes[1] can be described as complexes of singlet carbenes with transition-metal centers in which σ-donation from an sp^2 orbital is counterbalanced by π-donation into a vacant carbon p orbital (Scheme I).[1,2] In the archetypical carbene complexes of the group 6 metals, the carbene replaces an isolobal CO in a zero-valent hexacarbonyl to give a complex $[M(CO)_5\{C(ER_n)R'\}]$ (M = Cr, W; ER_n = heteroatomic substituent such as an alkoxy or amino group; R' = alkyl or aryl group) whose reactivity is dominated by the electrophilicity of the carbenoid carbon.[1] We recently became intrigued by the possibility that the π-accepting ability of heteroatomic carbenes might be sufficient to allow formation of stable complexes in which carbene ligands

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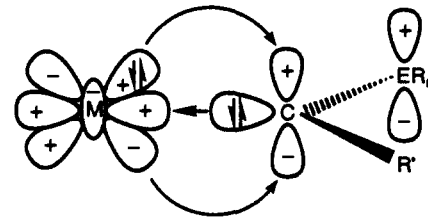
Scheme I. Coordination of the Ligand in a Fischer Carbene Complex[a]



[a] E = O, N, or S.

replaced a CO within a carbonylmetalate (such as $[Cr(CO)_5]^{2-}$) and by the further possibility that the increased back-donation to the unsaturated carbon would then change the carbenoid carbon into a nucleophilic center (similar to the unsaturated carbon in a Schrock alkylidene complex[3]). We now report (Scheme II) that dianionic carbene complexes such as $[Cr(CO)_4\{C(OMe)Ph\}]^{2-}$ ($\mathbf{1}^{2-}$) *are* synthetically accessible, and that the anticipated umpolung[4] *does* induce a fundamentally new reactivity pattern.

The synthesis of $\mathbf{1}^{2-}$ utilizes a strategy similar to that which we have used to prepare phosphine-substituted,[5] arene-substituted,[6] and cyclopentadienyl-substituted[7] carbonylmetalates: one CO of the neutral carbene complex $[Cr(CO)_5\{C(OMe)Ph\}]$ (**2**) is replaced with an alkylphosphine,[8] and the substituted complex is reduced with an alkali-metal naphthalenide at −78 °C. Low-valent Cr complexes with π-acceptor ligands tend to obey the 18-electron rule, and we anticipated that electron transfer would lead to phosphine loss and give a dianionic carbene complex.

Reductions utilized a mixture of *cis*- and *trans*-$[Cr(CO)_4(PBu_3)\{C(OMe)Ph\}]$ (**3**) prepared in 61% yield by PBu_3 substitution of **2**.[8] Solution IR data suggested that potassium 1-methylnaphthalenide reduction (2.0 equiv of a 0.2 M THF solution) of **3** (0.62 g, 1.27 mmol in 30 mL of THF) at −78 °C resulted in formation of the desired dianionic carbene complex, since the carbonyl absorptions of the starting material at 2018 (s) and 1900 (vs) cm^{-1} were replaced by absorptions at 1859 (s) and 1737 (s, br)[9] cm^{-1}, consistent with the formation of a highly reduced carbonyl complex. This dianion was isolated by addition of 18-crown-6 (18-C-6) to complex the K^+ cation. After 12 h at room temperature the solvent was removed under vacuum to give a red oil, which solidified when washed with hexanes (2 × 30 mL). The solid was redissolved in THF (30 mL), and 60 mL of hexanes was mixed with the solution to give a purple precipitate. ^{13}C and ^1H NMR spectra of the product[10] could be obtained by dissolving the purple solid in CD_3CN at 240 K and recording spectra of the solution at 243 K.[11] These spectra are consistent with the proposed formulation of the reduction product as $[Cr(CO)_4\{C(OMe)Ph\}]^{2-}$ ($\mathbf{1}^{2-}$) and include a ^{13}C resonance at δ 171.7

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(11) IR spectra of a cold (ca. 240 K) CH_3CN solution of $[K(18\text{-C-6})]_2$·**1** are very similar to those of a THF solution, with bands at 1850 (s) and 1720 (vs) cm^{-1}. NMR spectra were recorded at 240 K because the complex undergoes a reaction or isomerization in CH_3CN at room temperature to generate two new species with bands at 1890 (s) and 1755 (s) cm^{-1} and at 1860 (s) and 1720 (vs) cm^{-1}. When CD_3CN solutions of $[K(18\text{-C-6})]_2$·**1** are warmed above 240 K, changes in ^1H and ^{13}C NMR spectra are observed which correlate with the changes in the IR spectra.

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