which suggests the need of specific hydrogen bonding. Moreover, we[20] have shown in the 1:1 complex of clofazimine and DMF that intermolecular N(3)–H⋯O hydrogen bonds are formed to a ketonic oxygen acceptor atom. We hope to investigate the model further in future studies and shall attempt to isolate 1:1 complexes of clofazimine with polynucleotides.

Registry No. Clofazimine, 2030-63-9; isoclofazimine, 96728-46-0.

Supplementary Material Available: Hydrogen atom positional parameters, atomic thermal parameters, and observed and calculated structure amplitudes for all three molecules (48 pages). Ordering information is given on any current masthead page.

Solvent Isotope Effects on Formation of Protease Complexes with Inhibitory Aldehydes, with an Appendix on the Determination of Deuterium Fractionation Factors by NMR[†]
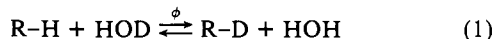
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Abstract: In both CCl_4 and water, equilibria of addition of thiols to acetaldehyde are strongly favored by the presence of deuterium at exchangeable positions, whereas equilibria of addition of hydroxylic compounds are hardly affected. To test the possibility of using solvent isotope effects for diagnosing the structure of enzyme–inhibitor complexes, we examined the influence of D_2O on the interaction of proteases with inhibitory aldehydes. Equilibrium constants for binding of inhibitory aldehydes showed enhancements in D_2O of 1.32 ± 0.12 for α-chymotrypsin and 2.65 ± 0.14 for papain, slightly in excess of those expected for covalent addition of an alcohol or thiol, respectively. In an Appendix, hydroxylic solutes are shown to alter the magnetic susceptibilities of solutions, resulting in systematic errors in apparent fractionation factors of their exchangeable protons as determined by conventional NMR procedures. As a result, apparent fractionation factors change with the orientation of the magnetic field. Corrected values for hydroxylic protons, determined by other methods, differ only slightly from unity.

Many enzymes that catalyze acyl-transfer reactions are reversibly inhibited by substrate-related aldehydes that can generate complexes resembling intermediates in substrate hydrolysis.[1,2] Other enzyme reactions, such as those involved in the oxidative phosphorylation of aldehydes, are believed to proceed by way of thiohemiacetal derivatives that serve as immediate substrates for dehydrogenation.[3] When the mechanism of action of such an enzyme has not been established, it would be of interest to know how an aldehyde inhibitor is bound: intact, or as an adduct formed by addition of an oxygen or a sulfur nucleophile. This information may also be useful in considering further improvements in inhibitor design. The form in which an aldehyde is bound to a specific enzyme can in principle be determined by using the diffraction of X-rays from single crystals of enzyme ligand complexes,[4] secondary deuterium isotope effects on equilibrium binding,[5,6] or nuclear magnetic resonance spectroscopy.[7-11] These methods require suitable enzyme crystals and highly purified aldehydes, incorporating isotopes at appropriate positions.

This paper explores the feasibility of an independent approach, based on differing affinities of reactants and products for deuterium at exchangeable sites.[12-14] If equilibrium constants (or fractionation factors, designated ϕ in eq 1) can be established for exchange of solute protons with deuterons from solvent water, then the effect of D_2O on the equilibrium constant of a reaction can be predicted (eq 2). The ratio of the equilibrium constant in D_2O

to the equilibrium constant in water is equivalent to the arithmetic product of the fractionation factors of the products divided by the arithmetic product of the fractionation factors of the reactants. Conversely, it should be possible to infer, from the influence of D_2O on the equilibrium binding of a competitive inhibitor, what changes in structure occur as an inhibitor is taken up by an enzyme. In cases where the structure of an enzyme–inhibitor complex is already available, it might also be possible to obtain structural information about states of the enzyme and inhibitor before reaction.

In attempting to evaluate this possibility, we became aware of apparent discrepancies between average values of 1.25 for fractionation factors of hydroxylic protons in *gem*-diols and hemiacetals determined by NMR[15] and values in the neighborhood

$$R–H + HOD \underset{}{\overset{\phi}{\rightleftharpoons}} R–D + HOH \qquad (1)$$

$$K_{eq}(D_2O)/K_{eq}(H_2O) = \Pi\phi(\text{products})/\Pi\phi(\text{reactants}) \qquad (2)$$

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of 1.08 estimated for *gem*-diols by comparison of equilibrium constants for hydration in water and in D_2O.[16-19] Recent experiments suggest that values obtained by NMR are subject to considerable uncertainty because of magnetic susceptibility effects and that there is no compelling evidence for hydroxylic proton fractionation factors much in excess of unity.[20] At the outset of the present work, it seemed desirable to determine the fractionation factor of a simple model hemiacetal, and we examined the influence of D_2O on the addition of methanol to acetaldehyde. The results led to a value of 0.93 ± 0.06 for the fractionation factor of the hydroxylic proton of acetaldehyde methyl hemiacetal in water and a solvent deuterium isotope effect (K_D/K_H) of 0.94 ± 0.06 on the equilibrium of hemiacetal formation. In contrast, equilibrium addition of sulfur nucleophiles to carbonyl compounds had been shown to be favored in D_2O by a factor of 2.25, as compared with its equilibrium in water.[16,17]

It remained to establish whether the larger solvent isotope effects typical of thiohemiacetal formation in water[16,17] might be sensitive to a change in the polarity of the surroundings in which thiol addition occurs. This possibility had been raised by earlier work, showing that thiol addition equilibria are markedly less favorable in chloroform than in water,[21] and by a report that the enolization of acetylacetone exhibits a pronounced equilibrium solvent isotope effect in water (K_D/K_H = 1.8) but not in methanol.[22] We therefore sought to determine whether deuterium isotope effects on alcohol and thiol addition were dependent on solvent environment.

These results suggested that it might be possible to distinguish between alternative structures of enzyme complexes with aldehydes, by comparing K_i values in water and in D_2O. The feasibility of this approach was tested by examining the influence of D_2O on apparent affinities of chymotrypsin and papain for inhibitory aldehydes, under conditions where their Michaelis constants and turnover numbers were insensitive to changing pH. For this purpose, aldehydes were chosen that had already been shown from other evidence to be bound as hemiacetals.[5-11]

Experimental Section

Materials. Ethanethiol (distilled before use) and benzene-d_6 (99 atom % excess) were purchased from Aldrich Chemical Co. Methanol-*OD* (99+ atom % excess) and deuterium oxide (99.9 atom % excess) were obtained from KOR Isotopes Co. Acetaldehyde, purchased from Eastman Kodak Co., was fractionally distilled through a Vigreaux column immediately before use. *p*-Nitroanilides of *N*-benzoyl-L-tyrosine and *N*-benzoyl-L-arginine, papain (2 times recrystallized), and α-chymotrypsin (3 times recrystallized) were purchased from Sigma Chemical Co. 5-Hydroxyvaleraldehyde was purchased from ICN Pharmaceuticals. 2-Methoxyethanethiol was prepared by published procedures[16,23] and redistilled twice before use (bp 110–112 °C). Ethanethiol-*SD* was prepared by stirring ethanethiol (25 mL) over deuterium oxide (6 mL, separate phase), to which a drop of dilute NaOD had been added, for 8 h at room temperature; this procedure was repeated 8 times, with NaOD omitted from the final washes. A similar method was used to prepare 2-methoxyethanethiol-*SD*, except that carbon tetrachloride was used to provide the bulk of the organic phase. *N*-Benzoylphenylalaninal (mp 143–144 °C after two crystallizations),[24] 2-benzamidoacetaldehyde,[25] and 2-acetamidoacetaldehyde[26] were prepared by published procedures.

Effects of D_2O on Methanol Addition to Acetaldehyde. Equilibria of addition of methanol to acetaldehyde in water and D_2O were determined by ultraviolet spectroscopy at 30 °C, using a Perkin-Elmer Model 124

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Table I. Proton Magnetic Resonances of Acetaldehyde, Thiols, and Thiohemiacetals in CCl_4

species	chem shift	species	chem shift
C*H₃*CHO	1.84 d	C*H₃*CHOHSCH₂CH₃	1.36 d
CH₃C*HO*	9.46 q	CH₃C*H*OHSCH₂CH₃	4.75 q
C*H₃*CH₂SH	1.14 t	CH₃CHOHSC*H₂*CH₃	2.53 m
CH₃C*H₂*SH	2.31 m	CH₃CHOHSCH₂C*H₃*	1.14 t
CH₃CH₂S*H*	1.04 t	C*H₃*CHOHSCH₂CH₂OCH₃	1.35 d
C*H₃*OCH₂CH₂SH	3.14 s	CH₃C*H*OHSCH₂CH₂OCH₃	4.74 q
CH₃OC*H₂*CH₂SH	3.26 t	CH₃CHOHSC*H₂*CH₂OCH₃	2.58 m
CH₃OCH₂C*H₂*SH	2.42 m	CH₃CHOHSCH₂C*H₂*OCH₃	3.34 m
CH₃OCH₂CH₂S*H*	1.28 t	CH₃CHOHSCH₂CH₂OC*H₃*	3.18 s

spectrophotometer equipped with a thermostated cuvette compartment. Methanol (or methanol-*OD*) was first weighed into a volumetric flask and then diluted to volume with H_2O (or D_2O) and adjusted to 4 °C. Fixed aliquots of a standard aqueous (or D_2O) solution of acetaldehyde (1.2 M) were mixed with varying amounts of these methanol stock solutions. Mixtures were then diluted to volume, transferred to cuvettes at 4 °C, and sealed with Teflon stoppers. Cuvettes were then adjusted to 30 °C, and, after a short interval to allow equilibration, the absorbance of the remaining acetaldehyde was determined at 278 nm as a function of increasing methanol concentration. Solutions to which no methanol had been added were used to determine the initial concentration of acetaldehyde. Equilibrium constants for hemiacetal formation were calculated from eq 3 and 4, where using values obtained by Kurz[18] for the

$$K_{hemi} = \frac{P}{(A_f)(M_i - P)} \quad (3)$$

$$P = A_o + A_o K_h - A_f - A_f K_h \quad (4)$$

A_o = acetaldehyde concentration in absence of methanol

A_f = acetaldehyde concentration in presence of methanol

M_i = initial methanol concentration

K_h = equilibrium constant for hydration

K_{hemi} = equilibrium constant for hemiacetal formation

equilibrium constant at 30 °C for hydration of acetaldehyde (0.914) and for the extinction coefficients of acetaldehyde in water (15.77) and in D_2O (15.62). The equilibrium constant for acetaldehyde hydration in D_2O (1.06) was calculated from the value in water and from the solvent isotope effect on hydration.[16]

Effects of Exchangeable Deuterium on Mercaptan Addition to Acetaldehyde in CCl_4. Equilibria of addition of thiols to acetaldehyde were determined in carbon tetrachloride by using a Bruker WM-250 NMR spectrometer operating at 250 MHz at 30 °C, from integrated peak intensities of product and reactants relative to a benzene standard. Proton resonances of reactants and products used in this study are shown in Table I. Equilibrium constants were obtained from the slopes of lines determined by plotting thiohemiacetal concentration divided by free acetaldehyde concentration, as a function of increasing thiol concentration.[21] After equilibration at 30 °C, mixtures contained acetaldehyde (0–0.8 M), thiohemiacetal (0–0.2 M), benzene as an integration standard (0.2 M), benzene-d_6 (2.0 mL) as a signal lock, and one of the following: ethanethiol-*SH* (0.1–0.6 M), ethanethiol-*SD* (0.1–1.0 M), 2-methoxyethanethiol-*SH* (0.04–0.20 M), or 2-methoxyethanethiol-*SD* (0.03–0.20 M), in a total volume of 10 mL. The content of unlabeled benzene in benzene-d_6, determined separately by using *p*-dioxane as an integration standard, was included in calculations of the benzene concentration. Deuterium enrichment in samples of thiols was determined by comparing the integrated peak intensities of carbon- and sulfur-bound protons. Infrared spectra of thiols, thiol adducts, methanol, and 5-hydroxyvaleraldehyde in CCl_4 were recorded with a Perkin-Elmer Model 710B spectrometer using a 0.1-mm cell.

Effects of D_2O on Inhibition of Proteases. At substrate concentrations much lower than K_m, a competitive inhibitor is expected to reduce the rate of an enzyme reaction by a fraction that corresponds to the inhibitor's fractional occupancy of the total number of enzyme active sites that are present. The effect of solvent deuterium on the dissociation constant of the competitive inhibitor *N*-benzoyl-L-phenylalaninal from α-chymotrypsin was accordingly determined at substrate concentrations much lower than the Michaelis constant, using the substrate *N*-benzoyl-L-tyrosine *p*-nitroanilide ($K_{m (app)}$ = 3.4 × 10⁻⁴ M).[27] Substrate hydrolysis

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Table II. Isotope Effects on the Addition of Nucleophiles to Carbonyl Compounds

nucleophile	carbonyl	solvent	N^a	$K_H{}^b$	$K_D{}^c$	atom fract deuterium	K_D/K_H
CH_3OH	CH_3CHO	water	21	0.543 ± 0.024	0.509 ± 0.021	0.99	0.94 ± 0.06
H_2O	CH_3CHO	water	5	0.85^d	0.99^d	1.0	1.16
CH_3CH_2SH	CH_3CHO	water	3	36^d			
$CH_3OCH_2CH_2SH$	CH_3CHO	water	5	32^d	72^d	1.0	2.25
$HOCOCH_2CH_2SH$	CH_3COCH_2F	water		0.170^e	0.330^e	0.99	1.95 ± 0.3
CH_3CH_2SH	CH_3CHO	water	3	36^d			
$HOCOCH_2CH_2SH$	CH_3COCH_2F	dioxane		0.083^e	0.190^e		2.29 ± 0.3
CH_3CH_2SH	CH_3CHO	CCl_4	5	0.419 ± 0.02	0.870 ± 0.06	0.96	2.08 ± 0.18
$CH_3OCH_2CH_2SH$	CH_3CHO	CCl_4	4	4.52 ± 0.4	10.1 ± 1.2	0.90	2.24 ± 0.38
α-chymotrypsin	N-benzoylphenylalaninal	water	15	$(4.90 \pm 0.20)^f \times 10^5$	$(6.48 \pm 0.53) \times 10^5$	0.99	1.32 ± 0.12
papain	2-acetamidoacetaldehyde	water	18	$(6.74 \pm 0.07) \times 10^3$	$(1.49 \pm 0.076) \times 10^4$	0.97	2.21 ± 0.11
papain	2-benzamidoacetaldehyde	water	18	$(1.10 \pm 0.037) \times 10^6$	$(2.84 \pm 0.11) \times 10^6$	0.97	2.65 ± 0.14

^{a} Number of determination in both H_2O and D_2O. ^{b} Equilibrium constant for nucleophilic addition to carbonyl compound in water expressed in units of M^{-1}. ^{c} Equilibrium constant in D_2O expressed in units of M^{+1}. ^{d} Reference 16. ^{e} Reference 17. ^{f} The values of association constants (slopes of lines in Figure 1) were determined as described in the Methods section. The standard deviation in the slope was considered equal to the square root of the sum of the squares of deviations of the data points from the calculated line divided by $(N - 2)$ (Bevington, P. R. "Data Reduction and Error Analysis for the Physical Sciences"; McGraw-Hill: New York, 1969; pp 113–118).
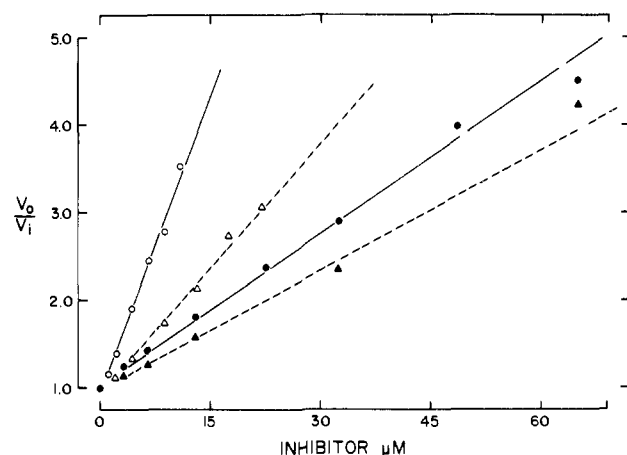

Figure 1. Ratios of uninhibited to inhibited initial reaction velocities plotted as a function of N-benzoyl-L-phenylalaninal concentration for the chymotrypsin-catalyzed hydrolysis of N-benzoyltyrosine p-nitroanilide in H_2O (closed triangles, dotted line) and in D_2O (closed circles, solid line). Ratios of uninhibited to inhibited initial reaction velocity are also plotted as a function of 2-benzamidoacetaldehyde concentration for the papain-catalyzed hydrolysis of N-benzoylarginine p-nitroanilide in H_2O (open triangles, dotted line) and in D_2O (open circles, solid line).

was monitored spectrophotometrically at 385 nm, in the absence and presence of varying concentrations of inhibitory aldehyde, in water and in D_2O (Figure 1). Reaction was initiated by addition of enzyme (10 μg in 10 μL of water)[28] to buffer (3 mL; 0.5 M sodium phosphate, pH 7.8), containing 0.1 M sodium chloride, substrate (9.1×10^{-6} M), and amino aldehyde (final concentration varying from 0 to 6.5×10^{-6} M) in dimethyl sulfoxide (10% v/v). Dissociation constants were obtained from the reciprocal of the slope of a plot of initial velocity of the uninhibited reaction divided by the initial velocity of the inhibited reaction, plotted as a function of increasing inhibitor concentration (Figure 1).

Effects of solvent D_2O on the dissociation constants of the competitive inhibitors 2-benzamidoacetaldehyde and 2-acetamidoacetaldehyde from papain were determined similarly, using the substrate N-benzoyl-L-arginine p-nitroanilide at concentrations far below the Michaelis constant ($K_m = 2.9 \times 10^{-3}$ M).[29] Papain was activated by incubation (15 min) at 20 °C in 0.1 M potassium phosphate buffer (pH 6.0) containing 0.01

M EDTA and 0.075 M β-mercaptoethanol and then passed down a Sephadex G15 column (58 cm × 1 cm) to remove small molecules. Substrate hydrolysis was monitored spectrophotometrically at 410 nm in the presence of varying concentrations of inhibitory aldehyde in both H_2O and D_2O. Reaction was initiated by addition of enzyme (50 μg in 50 μL of water) to buffer (2.40 mL; 0.1 M potassium phosphate, pH 6.0, 25 °C) containing potassium chloride (0.2 M), EDTA (1.0×10^{-6} M), substrate (1.63×10^{-4} M), and inhibitor (final concentration varying from 0 to 2.2×10^{-5} M for 2-benzamidoacetaldehyde and 0.0 to 7.0×10^{-3} M for 2-acetamidoacetaldehyde). D_2O solutions contained a final atom percent excess of 97. Dissociation constants were determined as described for chymotrypsin.

Results

Equilibrium addition of methanol to acetaldehyde was less favorable in D_2O than in water by a factor of 0.94 ± 0.06, corresponding to a fractionation factor of 0.93 ± 0.06 for 1-methoxyethanol. Equilibrium constants for addition of oxygen and sulfur nucleophiles, observed in the present study and in earlier work, are shown in Table II. The corresponding deuterium fractionation factors are shown in Table III.

To learn whether fractionation factors might be sensitive to a change in the polarity of the surroundings, solvent deuterium isotope effects on equilibrium constants for addition of mercaptans to acetaldehyde were determined in carbon tetrachloride. The results (Table II) for ethanethiol ($K_D/K_H = 2.08$) and 2-methoxyethanethiol ($K_D/K_H = 2.28$) were similar to isotope effects calculated at the same isotopic enrichment from previous data in aqueous solutions for ethanethiol ($K_D/K_H = 2.16^{16}$) and for 2-methoxyethanethiol ($K_D/K_H = 2.02^{16}$). Mixtures of methanol and acetaldehyde were not sufficiently stable to permit accurate determination of equilibria of single addition in CCl_4 solutions. However, comparison of infrared stretching and bending frequencies of CCl_4 solutions containing methanol or 5-hydroxyvaleraldehyde (more than 98% cyclic hemiacetal by NMR) allowed the deuterium isotope effect on hemiacetal formation in this solvent to be estimated. Stretching frequencies observed for methanol (3640 cm^{-1} for the free hydroxyl group and 3340 cm^{-1} for the hydrogen-bonded hydroxyl group) were similar to those observed for the cyclic hemiacetal (3600 cm^{-1} for the free hydroxyl group and 3400 cm^{-1} for the hydrogen-bonded hydroxyl group); spectra in the bending region appeared identical. Corresponding values for the expected effect of deuterium on the equilibrium constant, calculated according to the method of Bunton and Shiner,[30,31] ranged from 0.98 to 1.04 depending on stretching frequencies that were used in the calculation.

Infrared spectra in carbon tetrachloride of the adduct formed by reaction of acetaldehyde with 2-methoxyethanethiol exhibited a peak at 3425 cm^{-1} that did not disappear upon dilution, suggesting[32] the presence of an intramolecular hydrogen bond in this

(28) Although D_2O can influence protein structural equilibria (ref 13, 14), this appears to be a rare occurrence as indicated by NMR studies of protein structure and dynamics in D_2O (Venkatasubban, K. S.; Schowen, R. L. *CRC Crit. Rev. Biochem.* **1984**, *17*, 1–49). The possibility that structural effects might arise as a result of slow exchange processes should be minimized if, as in the present experiments, enzyme assays are carried out over a period of a few minutes following addition of a small aliquot of enzyme in H_2O to an assay mixture containing D_2O. More rapid exchange could lead to nonlinear reaction rates, but these have not been observed in the present experiments, or in earlier studies of other hydrolases (Wolfenden, R. *Biochemistry* **1969**, *8*, 2409–2412. Cohen, R. M.; Wolfenden, R. *J. Biol. Chem.* **1971**, *246*, 7561–7565).

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Table III. Isotopic Fractionation Factors of Alcohols, Thiols, and Aldehyde Adducts

compound	fract factor
methanol, CH_3OH	0.99[a]
1-methoxyethanol, $CH_3CHOHOCH_3$	0.93[b]
acetaldehyde hydrate, $CH_3CH(OH)_2$	1.08[c]
ethanethiol, CH_3CH_2SH	0.43[d]
1-(2-methoxyethylthio)ethanol, $CH_3CHOHSCHCH_3OCH_3$	1.0[e]

[a] Halford, J. O.; Pecherer, B. *J. Chem. Phys.* **1938**, *6*, 571. [b] Calculated from the solvent isotope effect on methanol addition (Table II) by using eq 1. [c] Calculated from the solvent isotope effect on the hydration of acetaldehyde (ref 16–19) by using eq 2. [d] Hobden, F. W.; Johnson, E. F.; Weldon, L. H. P.; Wilson, C. J. *J. Chem. Soc.* **1939**, 61–67. [e] Calculated from the solvent isotope effect on thiohemiacetal formation (ref 16) by using eq 1.

thiohemiacetal. No such peak was observed in spectra of the ethanethiol adduct. In apparent accord with this interpretation was the finding that the equilibrium constant for addition of ethanethiol to acetaldehyde was reduced to a greater extent on transfer from water to carbon tetrachloride than was the equilibrium constant for addition of 2-methoxyethanethiol (Table II).

Aldehyde inhibitors of papain and chymotrypsin were more tightly bound in D_2O than in water, but this effect was much more pronounced for papain than for chymotrypsin. Table II shows apparent association constants (reciprocals of K_i values) for aldehyde inhibitors of papain and chymotrypsin, measured under conditions where dissociation constants and kinetic properties of these enzymes were insensitive to changing pH.[24,33,34] Dissociation constants were determined at pH 7.8 for chymotrypsin, for which aldehyde binding depends on the conjugate base of one group with an apparent pK_a of 4.0,[24] and at pH 6.0 for papain, for which aldehyde binding shows a bell-shaped pH dependence with apparent pK_a values of 4.3 and 8.6.[34] Initial rates were determined in the absence and presence of increasing concentrations of inhibitor, at substrate concentrations far below their respective K_m values (see Experimental Section). Plots of uninhibited velocity divided by inhibited velocity, as a function of changing inhibitor concentration, produced straight lines intersecting the ordinate at 1.0 (Figure 1). The solid lines in Figure 1 show results obtained in D_2O, and the broken lines show results obtained in water where inhibition was less effective. After calculation of the concentration of free aldehyde from the equilibrium constant for hydration[5,24,26] and correction for the small effect of solvent deuterium on the hydration of aldehydes (Table III and ref 16–19), apparent ratios of enzyme–inhibitor association constants (K_D/K_H) were 1.32 ± 0.12 for *N*-benzoylphenylalaninal + chymotrypsin (Figure 1, closed symbols), 2.65 ± 0.14 for 2-benzamidoacetaldehyde + papain (Figure 1, open symbols), and 2.21 ± 0.11 for 2-acetamido-acetaldehyde + papain (data not shown).

Discussion

The possibility that fractionation factors might be sensitive to changes in the polarity of the surroundings was raised by an earlier report that enolization of acetylacetone exhibits a large deuterium solvent isotope effect in water but not in methanol.[22] The present results show that this is not the case for equilibria of acetal or thiohemiacetal formation. Other studies have shown that the effect of solvent deuterium on equilibrium addition of both water and 2-mercaptopropionate to fluoroacetone is affected very little by transfer of the reaction from water to dioxane[17] (Table II). The present findings regarding methanol addition also appear consistent with our earlier observation that the equilibrium addition of methanol to acetaldehyde is hardly affected by transfer of the reaction from water to chloroform;[21] evidently the aggregate strength of hydrogen-bonding interactions with solvent water are similar for reactants and products of methanol addition.

Deuterium solvent isotope effects observed here for enzyme inhibition are in the approximate ranges expected if aldehydes are bound by chymotrypsin and papain as hemiacetals and thiohemiacetals, respectively. For papain, a similar value (with a substantial compounded error) can be estimated by comparing rate constants reported in both water and D_2O for the onset of, and release from, aldehyde inhibition.[34] Solvent isotope effects on the binding of 2-benzamidoacetaldehyde and *N*-benzoyl-phenylalaninal appear, nonetheless, to be significantly larger than values expected from the model reactions (Table 11 and Figure 1). In nonaqueous solvents, primary isotope effects on hydrogen bond formation are nearly unity for bonding between alcohols, between thiols, between amines,[35] and between amines and phenols.[36] These results have been interpreted as suggesting that in free solution, reductions in bending motions resulting from hydrogen bond formation may be compensated by increases in stretching motions.[14] It seems possible that multiple binding interactions in a highly ordered active site could lead to uncompensated reductions in both bending and stretching modes of a hemiacetal hydroxyl group in a strong complex. This might be expected to produce a fractionation factor greater than unity, and a solvent isotope effect larger than that observed for the corresponding reaction in free solution. The complex formed by 2-acetamidoacetaldehyde with papain is much looser and is only 20-fold more stable than a typical model thiohemiacetal (Table 11, ref 37). In this case, where other interactiions appear to be of relatively minor importance, the effect of solvent deuterium on enzyme–inhibitor association did not differ significantly from that observed for model reactions in free solution.

An alternative explanation for the solvent isotope effect on aldehyde association with proteases being greater than predicted is that aromatic compounds have an unfavorable free energy of transfer from water to D_2O. Accordingly, this type of compound may be more likely to self-associate in D_2O than in water, so that binding of aromatic portions of inhibitory aldehydes to enzyme binding sites, also containing aromatic residues, might be enhanced in D_2O relative to water. Another possible explanation of results obtained with chymotrypsin is that an isotope effect in excess of unity arises from a reactant state fractionation factor estimated in the literature as 0.7–0.8.[40] However, it is unclear whether that value is for a single histidine proton (which would not be expected to contribute to the solvent isotope effect on hemiacetal formation) or for both a histidine and a serine proton (of which only the serine could reasonably be expected to contribute to the observed solvent isotope effect).

The present results are not easy to reconcile with the possibility that active papain may exist in the ground state as a thiolate-imidazolium ion pair, mainly[41–44] or in part.[45–47] Thiohemiacetal formation is expected to show a solvent isotope effect of 2.25 on enzyme–inhibitor association, and values this large or larger have been observed in the present experiments. The fractionation factor of an imidazolium group is approximately unity,[12,13] so that

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Table IV. Predicted and Observed Effects of Deuterium Substitution on Equilibrium Association of Inhibitory Aldehydes with Proteases

| | apparent K_D/K_H[a] | |
effects for binding as:	aldehyde deuterium substit.	solvent deuterium substit.
predicted		
intact aldehyde	0.75[b]	0.9 ± 0.02[d]
hydrate	1.0[b]	1.0 ± 0.02[d]
hemiacetal	1.0[b]	0.8 ± 0.05[e]
thiohemiacetal	0.9[b]	2.0 ± 0.25[f]
observed		
α-chymotrypsin		1.15 ± 0.1[e,g]
papain	0.9[b,c]	2.3 ± 0.1[c,e]

[a] Equilibrium constants not corrected for hydration or deuterium isotope effects on hydration. [b] Reference 26. [c] 2-Benzamidoacetaldehyde. [d] This value was predicted from the average of several values of K_D/K_H for aldehyde hydration (ref 16–19), and the error was estimated from the standard deviation in those values. [e] This work. [f] Reference 16. [g] *N*-Benzoyl-L-phenylalaninal.

thiohemiacetal formation from a thiolate–imidazolium ion pair would not be expected to change in the presence of solvent deuterium. Accordingly, these results appear more readily understandable in terms of a ground state involving neutral thiol and imidazole groups. Alternatively, the fractionation factor could be reduced by the existence of a symmetrical hydrogen bond in which the barrier to proton transfer is low.[48]

In the absence of perturbing influences, effects of solvent deuterium on enzyme–inhibitor association appear to be most useful for distinguishing thiohemiacetal formation (apparent $K_D/K_H \geq 2$) from alternative structural possibilities for which K_D/K_H is expected to be near unity. In contrast, effects of deuterium substitution in the aldehyde appear to be more useful for distinghishing binding of the aldehyde intact (appareant K_D/K_H = 0.75), from alternative possibilities for which K_D/K_H is expected to be near unity (Table IV, ref 5). These methods can be used in combination to determine whether an aldehyde is bound intact or as a sulfur or an oxygen adduct and require only small amounts of enzyme in an incomplete state of purity.

Appendix

The interpretation of solvent isotope effects on rates and equilibria depends on the availability of equilibrium constants for exchange of solute protons with deuterons from solvent water or fractionation factors (0 in eq 1a). For any reaction, the effect

$$S-H + HOD \underset{}{\overset{\phi}{\rightleftharpoons}} S-D + HOH \qquad (1a)$$
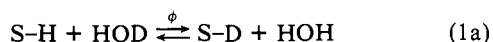
of solvent D_2O on the overall equilibrium constant is equal to the arithmetic product of the fractionation factors of reaction products divided by the arithmetic product of the fraction factors of the reactants.[12,13] During an investigation of possible uses of deuterium solvent isotope effects in investigating structures of enzyme–inhibitor complexes, we became aware of differences between average values of 1.25 for fractionation factors of hydroxylic protons in *gem*-diols and hemiacetals determined by NMR[15] and values nearer unity calculated from solvent isotope effects on equilibrium addition of water and alcohols to aldehydes (1.09,[19] 1.08,[16] 1.06,[18] and 1.08[17] for for water addition; 0.95 ± 0.06[49] for methanol addition).

Deuterium fractionation factors of solutes can be determined by NMR spectroscopy, by monitoring the chemical shift of exchangeable protons, relative to an external reference, as a function of increasing mole fraction of solute in water and in D_2O of known isotopic enrichment.[50–52] If *n* is the atom fraction of deuterium
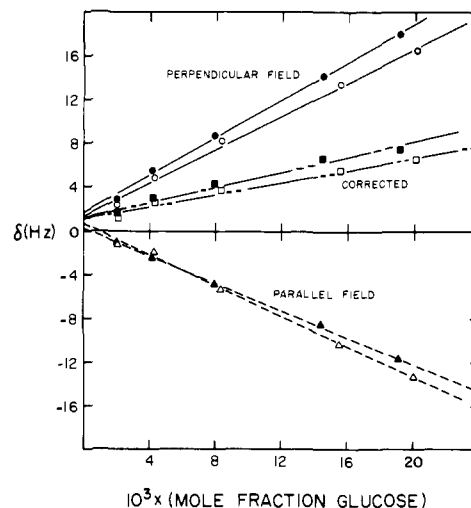

Figure 2. Changes in chemical shift (Hz), relative to an external standard of pure solvent, plotted as a function of moles of glucose hemiacetal sites divided by moles of total exchangeable sites. Data collected in H_2O (solid circles, squares and triangles) and D_2O (open circles, squares and triangles) using perpendicular (circles) and parallel (triangles) magnetic fields and data corrected for differing sample and reference magnetic susceptibilities (squares) are shown.

in D_2O, then the fractionation factor can be calculated from the slopes of the two plots by using eq 2a.

$$\frac{slope_{(H_2O)}}{slope_{(D_2O)}} = 1 - n + n\phi \qquad (2a)$$

The purpose of this note is to draw attention to a potential difficulty in such measurements that does not seem to have been considered before. The difference between the chemical shift of a sample and an external reference is affected by any difference between magnetic susceptibilities of the sample (κ_s) and the reference (κ_r).[53] Equation 3a expresses this relationship for a cylindrical sample with its axis perpendicular to the applied field.

$$\delta_{true} = \delta_{measured} - \tfrac{2}{3}\pi \times 10^6(\kappa_s - \kappa_r) \qquad (3a)$$

Differences between magnetic susceptibilities of water and D_2O do not influence the slopes of plots that are used in applying eq 2a, because their concentrations remain virtually constant as the concentration of the solute changes.[54] The possibility remains that changing concentrations of the solute could alter magnetic susceptibilities of samples. If such effects were significant, they would introduce a systematic error into apparent differences in chemical shift between the sample and an external reference, resulting in an error in the apparent fractionation factor. In accord with this possibility, it has been noted that the chemical shift of water in pyridine–water mixtures differes anomalously from that of an external reference consisting of pure water.[55]

Effects arising from differences between magnetic susceptibilities of a sample and an external reference should be detectable by comparing chemical shifts determined by using a spectrometer with a magnetic field perpendicular to the axis of the spinning sample tube ($\delta \perp$), with chemical shifts determined by using an instrument with a magnetic field parallel to the sample tube ($\delta^{||}$).[53,56,57] Chemical shifts free of magnetic susceptibility effects (δ_{true}) are related to these observed chemical shifts by eq 4a.

$$\delta_{true} = \tfrac{1}{3}(\delta^{||} + 2\delta \perp) \qquad (4a)$$

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Table V

solute	concn.[a] range	atom fract. deuterium	slope perpend. field[b]	slope parallel field[b]	slope corr.[c]
glucose	0.002-0.02	0.0	164 ± 34	-123 ± 4	68.6
glucose	0.002-0.02	0.90	154 ± 26	-138 ± 21	56.8
α-methyl glucoside	0.002-0.02	0.0	594 ± 26	996 ± 157	726
α-methyl glucoside	0.002-0.02	0.93	372 ± 91	1145 ± 79	630

[a] Moles of one solute exchangeable site/moles of total exchangeable sites. [b] Slope of plot of chemical shift of exchangeable proton resonance, relative to an external reference of pure solvent, as a function of solute concentration, Hz/(moles of one solute exchangeable site/moles of total exchangeable sites). Positive values indicate upfield shifts of sample resonance relative to external reference. [c] Data corrected (using eq 2) for the difference between the magnetic susceptibilities of sample and reference solutions, Hz/(moles of one solute exchangeable site/moles of total exchangeable site).

Figure 2 shows the results of such an experiment, comparing chemical shifts obtained for glucose in two different spectrometers. In these experiments, solutions were prepared gravimetrically from α-D-glucose and transferred to the outer cell of a coaxial tube; pure solvent (H_2O or D_2O of atom fraction deuterium, n) was then added to the internal cell, and the tube was sealed with parafilm and allowed to equilibrate overnigth at 34 °C. Chemical shifts were determined relative to the external reference, first by using a Bruker WM-250 spectrometer operating at 250 MHz and 34 °C and then by using a Varian EM-390 spectrometer operating at 90 MHz and at 34 °C. For comparison of results, chemical shifts were corrected to a common operating frequency of 90 MHz. Figure 2 shows chemical shifts that were observed for exchangeable protons of glucose solutions in H_2O (closed symbols) and D_2O (open symbols). Results obtained by using a spectrometer employing a permanent magnet with a perpendicular field (circles) are quite different from those obtained with a superconducting magnet with a field parallel to the sample (triangles), indicating

that magnetic susceptibility effects are pronounced. Table V compares the slopes obtained with glucose and with α-methyl glucoside, and it can be seen that magnetic susceptibility effects of these compounds differ in both a qualitative and a quantitative sense.

In principle, true fractionation factors can be computed from these results by using eq 2a and 4a. However, compounded errors in the resulting values (1.23 ± 0.28 for glucose and 1.17 ± 0.16 for α-methyl glucoside) are too great to be useful in deciding whether they differ significantly from unity. How likely does it seem that fractionation factors of individual hydroxyl groups in thes compounds differ from unity? The values above represent complex averages of values for all the exchangeable protons in these compounds, which include primary and secondary alcoholic groups and, in the case of glucose, a hemiacetal hydroxyl group. Direct measurements indicate a value of 0.99 for methanol.[58] A solvent isotope effect of 0.94 ± 0.06 on methanol addition to acetaldehyde[49] suggests a fractionation factor of 0.95 ± 0.06 for the resulting hemiacetal. Equilibria of covalent hydration of 2-acetyl-3,4-dimethylthiazolium ion[59] and of addition of one and two water molecules to pteridine[60] also indicate fractionation factors indistinguishable from unity. Slightly higher values, averaging 1.08[16-19] are calculated for the fractionation factors of protons in *gem*-diols formed from aliphatic aldehydes. With these last exceptions, it seems that NMR evidence for hydroxyl fractionation factors in excess of unity may not be well founded and that values obtained by other methods may be more reliable.

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Communications to the Editor

Valence Delocalization in Mixed-Valence 1',6'-Diiodobiferrocenium Triiodide

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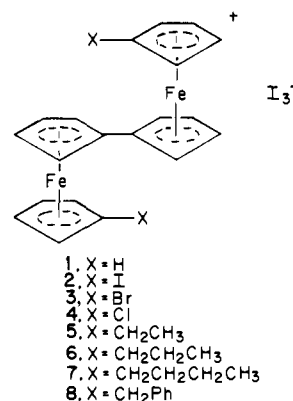David N. Hendrickson,*[1] and Cortlandt G. Pierpont*[2]

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Discoveries made over the past 2 years have contributed to a much clearer understanding of factors that control intramolecular electron transfer in mixed-valence transition-metal complexes. In a very recent paper[3] it was shown that for the oxo-centered mixed-valence complex [Fe$_3$O(O$_2$CCH$_3$)$_6$(4-Et-py)$_3$](4-Et-py), where 4-Et-py is 4-ethylpyridine, a transformation in the solid-state structure from statically disordered at low temperatures to dynamically disordered at high temperatures dramatically affects the intramolecular electron-transfer rate. Similar observations have now been made on several other mixed-valence trinuclear

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iron acetate complexes.[4] In this paper we report observations on biferrocenium triiodide (1), which has previously been reported[5]

1. X = H
2. X = I
3. X = Br
4. X = Cl
5. X = CH$_2$CH$_3$
6. X = CH$_2$CH$_2$CH$_3$
7. X = CH$_2$CH$_2$CH$_2$CH$_3$
8. X = CH$_2$Ph

to be valence localized at 300 K, and the X-ray structure of 1',6'-diiodobiferrocenium triiodide (2), which is valence delocalized in the range 300-4.2 K.

The series of substituted biferrocenium complexes listed above will provide important information on intramolecular electron

(4) Oh, S. M.; Hendrickson, D. N., unpublished results.