in volume that results from replacing nearby, spatially constrained amino acid residues by freely compressible solvent. The native protein conformation would not undergo appreciable decrease in volume with pressure because the rigidity of the backbone prevents nearby residues from coming together. On the other hand, these residues will contribute to the decrease in volume as they come in contact with solvent upon unfolding. A quenched derivative with a very short link could be expected, in the limit, to behave like the protein, increasing its fluorescence as the pressure is raised beyond a certain value. The short-linked flavinyl peptide investigated does not have that behavior, but its much poorer compressibility as compared with the larger peptide, or with FAD, indicates that the linking covalent frame produces the expected effects. The synthesis of appropriate molecular models, which would undergo unfolding under pressure like the proteins, seems therefore a distinct possibility.

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Antiproteolytic Aldehydes and Ketones: Substituent and Secondary Deuterium Isotope Effects on Equilibrium Addition of Water and Other Nucleophiles†

Charles A. Lewis, Jr.,‡ and Richard Wolfenden*

ABSTRACT: Equilibrium constants for hydration of ketones, in dilute D_2O solution at 34 °C, observed by proton magnetic resonance under conditions of slow exchange, were acetone 0.002, chloroacetone 0.08, 1,3-dichloroacetone 4.17, bromoacetone 0.07, and 1,3-dibromoacetone (an inhibitor of papain) 1.85. Neither acetamidoacetone nor *N,N*-diacetylaminoacetone showed evidence of appreciable hydration in dilute aqueous solution, nor was any hydrate detectable in solutions of tosylglycine chloromethyl ketone. Substitution of acetaldehyde with acylamido substituents, as in several potent reversible inhibitors of papain, was found to enhance its equilibrium constant for covalent hydration by an order of magnitude; these inhibitors are about 90% hydrated in dilute aqueous solution, and their affinity for proteases may have been underestimated accordingly. The effects of deuterium substitution at C-1 of acetaldehyde, on equilibrium addition of oxygen and sulfur nucleophiles, are substantial and vary with the nature of the nucleophile. These isotope effects may be useful as a means of distinguishing between alternative structures of complexes formed between enzymes and aldehydes.

Carbonyl compounds bearing appropriate specificity determinants are strong inhibitors of proteolytic enzymes and amidases. Halomethyl ketones inactivate proteases of the chymotrypsin type by alkylating an active site nucleophile (Schoellman and Shaw, 1962). Further reaction may ensue, as in alkylation of subtilisin BPN, which is accompanied by the formation of an internal hemiacetal involving a serine residue at the active site (Poulos et al, 1976). Halomethyl ketones are sometimes found to act as very strong *reversible* inhibitors, as in the case of certain aminopeptidases (Birch et al., 1972; Kettner et al., 1974). Several explanations could be offered for these latter observations, but it appears likely that they may be related to the ability of such compounds to undergo reversible addition of nucleophiles. A strong inhibitor of cholinesterase has recently been designed on this basis (Dafforn and Kerr, 1976).

Aldehydes, related in structure to carboxylic acids released by substrate hydrolysis, have also been found to serve as highly effective inhibitors of several hydrolytic enzymes, including papain (Westerik and Wolfenden, 1972), elastase (Thompson, 1973), L-asparaginase (Westerik and Wolfenden, 1974), and a bacterial amidase (Findlater and Orsi, 1973). These aldehydes were designed to form adducts that might resemble intermediates in substrate transformation. Umezawa and his

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colleagues have discovered in nature a group of oligopeptides terminated by L-argininal (Aoyagi et al., 1969; Maeda et al., 1971, Suda et al., 1972; Ito et al., 1972). The antibiotic effects of these oligopeptides may be based on similar inhibition.

These inhibitors form enzyme complexes that may be structurally related to intermediates in substrate transformation, and the structures of these complexes are of mechanistic interest in addition to their possible bearing on drug design. Many simple carbonyl compounds undergo reversible covalent addition of water in dilute aqueous solution (see Bell (1966) for references). To permit proper evaluation of the affinity of enzymes for the form of the inhibitor with which they combine, it seemed desirable to obtain information about the state of hydration of some of the major protease inhibitors, and to examine the effect of deuterium substitution in aldehydes as a possible means of distinguishing between alternative structures of their inhibitory complexes with enzymes.

Materials and Methods

Acetaldehyde (Eastman) was distilled at 21 °C through a column packed with glass helices. Acetaldehyde-*1-d* (Merck, Sharp and Dohme, Canada) was found to be free of spectroscopically detectable contaminants, and was used without further purification. *N*-Acetyl-L-phenylalanylaminoacetaldehyde diethyl acetal (Westerik and Wolfenden, 1972), *N*-acetylaminacetone (Wiley and Borum, 1948), *N,N*-diacetylaminoacetone (Wiley and Borum, 1948), and 1,3-dibromoacetone (Weygand and Schmeid-Koworski, 1949) were prepared according to published procedures. L-Leucine chloromethyl ketone (Kettner et al., 1974) was a gift from Dr. Retford Berko. 2-Mercaptoethanol (Sigma), chloroacetone (Fluka), 1,3-dichloroacetone (Fluka), and bromoacetone (Fluka) were obtained commercially and redistilled before use.

N-Acetamidoacetaldehyde was prepared by mixing aminoacetaldehyde dimethyl acetal (105 g) (Aldrich) with water (300 mL) and acetic anhydride (205 g). The mixture was stirred for 1 h and then stored in the cold overnight. Solvents were removed by rotary evaporation and vacuum distillation at 105 °C/0.9 mm yielded 25.5 g (17.4% of theory) of *N*-acetylglycinal dimethyl acetal: NMR[1] (60 MHz, neat), 1.88 ppm (s, 3 H), 3.20 ppm (t, J = 6 Hz, 2 H), 4.38 ppm (t, J = 6 Hz, 1 H), 8.00 ppm (t, J = 6 Hz, 1 H). The free aldehyde was generated by hydrolysis of the acetal (3.0 g) in D_2O (35 mL) with Dowex 50X-X8-H^+ (15 g) stirred for 27 h. After filtration and removal of solvent under vacuum, the yield of *N*-acetylglycinal was nearly quantitative; NMR (100 MHz, Me_2SO-d_6) showed a mixture of aldehyde and hydrate. Aldehyde: 1.91 ppm (s, 3 H), 3.89 ppm (*d*, J = 0.7 Hz, 2 H), 9.45 ppm (t, J = 0.7 Hz, 1 H). Hydrate: 1.83 ppm (s, 3 H), 3.00 ppm (d, J = 5.3 Hz, 2 H), 4.77 ppm (t, J = 5.3 Hz, 1 H).

Ultraviolet spectroscopy was performed using either a Zeiss PMQII spectrophotometer or a Perkin-Elmer Model 124 spectrophotometer, both instruments with thermostatically controlled cuvette compartments maintained at 25.0 ± 0.2 °C.

Analytical NMR spectra were recorded at 60, 90, and 100 MHz on JEOL C60HL, Varian EM-390, and Varian XL-100-12 spectrometers, observing proton nuclei. Chemical shifts are indicated on the chemical shift scale, downfield from internal reference signals provided by Me_4Si or TSP.

[1] Abbreviations used: NMR, nuclear magnetic resonance; TSP, sodium trimethylsilapropanoate-d_4; TGCK, tosylglycine chloromethyl ketone; TLCK, tosyllysine chloromethyl ketone.

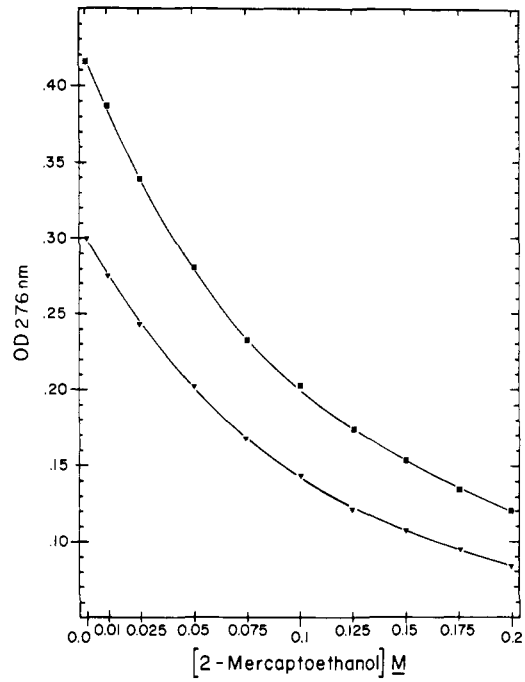

FIGURE 1: Changes in ultraviolet absorption of (■) acetaldehyde and (▼) acetaldehyde-*1-d* (each 5 × 10^{-2} M in water) with addition of 2-mercaptoethanol. Solid lines are calculated for equilibrium constants for mercaptan addition: K = 33.2 for acetaldehyde and K = 41.6 for acetaldehyde-*1-d*, based on the concentration of unhydrated aldehyde (see text), at 25 °C.

For measurements of equilibria, spectra of aldehydes and ketones (approximately 5 × 10^{-2} M except in the special case of acetone) were recorded in D_2O solutions at 22–23 °C on a Varian XL-100-12 Fourier Transform NMR spectrometer observing proton nuclei, or on a Varian EM-390 NMR spectrometer observing proton nuclei at 34 °C, as indicated in Table II. Spectra with high signal-to-noise ratios were required to obtain accurate integrals. Sets of five integrals were recorded over the peaks listed in Table I, maximizing the integral on the largest peak of each pair of resonances. Integrals were found to agree within 2% on the XL-100, 5% on the EM-390.

The equilibrium constant for hydration was calculated by substituting the appropriate values of integrals into eq 1.

$$K_{\text{hydration}} = \frac{(\text{hydrate integral})}{(\text{aldehyde integral})} \quad (1)$$

When two pairs of peaks were used to determine hydration equilibria, values calculated for $K_{\text{hydration}}$ were averaged to yield the value reported in Table II.

Equilibrium constants for addition of 2-mercaptoethanol to acetaldehyde were determined as described by Lienhard and Jencks (1966). The equilibrium constant for addition of 2-mercaptoethanol to acetaldehyde and acetaldehyde-*1-d* was determined by monitoring the absorbance at 276 nm of an aqueous solution (5 × 10^{-2} M) of the aldehyde in the presence of varying concentrations of thiol (0.01–0.20 M). Five replicate samples were measured at each thiol concentration, yielding results typified by those shown in Figure 1. The equilibrium constant, corrected for hydration, was calculated using eq 2 (Lewis and Wolfenden, 1973).

$$K_{\text{addition}} = \frac{(\text{adduct})}{(\text{free aldehyde})(\text{free thiol})} = \frac{T - A - (A \cdot K_{\text{hydration}})}{A(M - T + A + (A \cdot K_{\text{hydration}})} \quad (2)$$

TABLE I: Proton NMR Resonances Used in Measurements of Hydration Equilibria in D_2O.

Aldehydes	Chemical shifts (ppm) Aldehyde	Hydrate
Acetaldehyde		
2-CH_3	2.25	1.33
Acetaldehyde-*1-d*		
2-CH_3	2.24	1.33
Acetamidoacetaldehyde		
1-CHO,-$CH(OH)_2$	9.54	5.08
2-CH_2	4.76	3.28
Benzamidoacetaldehyde		
1-CHO,-$CH(OH)_2$	9.41	5.00
2-CH_2	4.13	3.23
N-Acetyl-L-phenylalanylaminoacetaldehyde		
2-CH_2 (glycyl)	3.88	3.23
Ketones	**Ketone**	**Hydrate**
Acetone[a]		
1-CH_3	2.30	1.56
^{13}C satellite	1.71	
Chloroacetone		
3-CH_3	2.30	1.50
1-CH_2	4.50	3.60
Bromoacetone		
3-CH_3	2.37	1.55
1-CH_2	4.30	3.55
1,3-Dichloroacetone		
CH_2	4.55	3.77
1,3-Dibromoacetone		
CH_2	4.43	3.70
N-Acetylaminoacetone[b]		
CH_3 (acetone)	2.05	
CH_3 (acetyl)	2.25	
CH_2	4.13	
N,N-Diacetylaminoacetone[b]		
CH_3 (acetone)	2.20	
CH_3 (acetyl)	2.35	
CH_2	4.65	

[a] The hydration of acetone was measured by comparison of the peak heights of the ^{13}C satellite and the hydrate peak, as performed earlier by Hine and Redding (1964). [b] These compounds do not exhibit peaks that are assignable to the hydrate, and are assumed to be less than 1% hydrate in aqueous solution.

TABLE II: Equilibrium Constants for Hydration of Aldehydes and Ketones Measured by Proton NMR in D_2O Solutions.

Aldehydes	$K_{hydration}$
Acetaldehyde[a]	1.38
Acetaldehyde-*1-d*[a]	1.89
Acetamidoacetaldehyde[a]	11.23
Benzamidoacetaldehyde[a]	11.82
N-Acetyl-L-phenylalanylglycinal[a]	7.90
Ketones	
Acetone[a,b]	0.002
Chloroacetone[c]	0.08
Bromoacetone[c]	0.07
1,3-Dichloroacetone[c]	4.17
1,3-Dibromoacetone[c]	1.85
N-Acetylaminoacetone[c,d]	≤0.01
N,N-Diacetylaminoacetone[c,d]	≤0.01

[a] Measured on the XL-100-12 at 22–23 °C, with an estimated error of ±2%. [b] Obtained by comparison of hydrate signal to ^{13}C satellite of ketone peak as performed by Hine and Redding (1964), and in agreement with their results. [c] Measured on the EM-390 at 34 °C with an error of ±5%. [d] Since these compounds did not exhibit resonances due to the formation of a hydrate, an upper limit of 1% hydrate was assumed in order to calculate the equilibrium constant shown above.

where

T = total aldehyde in solution

M = total thiol in solution

A = free aldehyde

Results

Table I shows the chemical shifts of resonances used in determining equilibria of hydration of aldehydes and ketones in D_2O. In all cases it proved convenient to use, for integration, the peaks assigned to methyl or methylene protons adjacent to the aldehyde carbon. In the case of acetone, in which the abundance of hydrate in dilute aqueous solution is so much lower than that of the hydrate that their integrals cannot be compared (due to the dynamic range limitations of the spectrometer), the 0.55% (^{13}C) satellite peak of the methyl protons was used as an integration standard for comparison with the hydrate. Unlike the other measurements, that were made in dilute (0.05 M carbonyl compound) solution, the acetone measurements were made in relatively concentrated (30% by volume) solution in D_2O in order to observe the weak signal from the hydrate. In all cases exchange was slow on the NMR time scale.

The results, tabulated in Table II, show that acylamido substituents, such as those present in several aminoacetaldehyde derivatives that inhibit papain, substantially enhance the equilibrium of hydration of acetaldehyde. Halogen substituents produce even larger enhancements in the equilibrium of hydration of acetone, and the protease inhibitor 1,3-dibromoacetone is more than 50% hydrated in dilute aqueous solution. Neither acetamidoacetone nor *N,N*-diacetylaminoacetone exhibited appreciable hydration, nor was any hydrate detected in samples of leucine chloromethyl ketone, tosylglycine chloromethyl ketone (TGCK) or tosyllysine chloromethyl ketone (TLCK). Spectra of the last three compounds were rather poorly resolved, and in the case of TGCK the insolubility of the compound in water required that spectra be determined in mixtures of deuterated water and deuterated dimethyl sulfoxide.

A significant discrepancy was found between hydration equilibrium constants for acetone derivatives and those reported in an earlier study by Bell and McDougall (1960). The probable source of difficulty is that the earlier study relied on the assumption that the ultraviolet absorption of the free carbonyl compound simply diminishes as hydrate is formed, so that an equilibrium constant for hydration can be calculated by comparing the observed extinction coefficient of the carbonyl compound in hexane and in water. This procedure is only justified when there is evidence that the change of solvent does not significantly affect the extinction coefficient of individual species or their band shapes. In fact it was observed that, when ultraviolet spectra of freshly distilled chloroacetone were recorded in hexane and water, marked changes in band shape occur, especially in the vicinity of 265 nm where measurements were made in the earlier study. In the present study, resonances of the hydrate methyl and methylene protons were found to shift 0.8 ppm upfield from corresponding peaks of the ketone, comparable to shifts occurring when acetaldehyde was converted to its hydrate, and permitted direct measurements of the relative concentrations of both species. Ratios of integrals yielded for chloroacetone a hydration equilibrium constant of

TABLE III: Deuterium Effect on Equilibrium Constants for Hydration and Thiohemiacetal Formation.

Equilibrium constant	K_{H_2O}[a]	K_{SH}[b]
Acetaldehyde	1.38 ± 0.02	33.2 ± 1.3
Acetaldehyde-*1*-*d*	1.89 ± 0.03	41.6 ± 1.7
K_D/K_H	1.37 ± 0.03	1.25 ● 0.06

[a] Determined by proton NMR (Table II) at 22–23 °C. Equilibrium constant with total water activity taken as unity. [b] Determined by UV at 25 °C. Equilibrium constant for free aldehyde (total aldehyde corrected for hydration) as described in text.

0.08, in reasonable agreement with an ^{17}O NMR measurement reported by Greenzaid et al. (1967), and substantially smaller than the value of 0.54 reported by Bell and McDougall (1960). Other haloketones in Table II also exhibit much smaller hydration equilibrium constants than those reported in the earlier work.

Hydration of acetaldehyde showed a pronounced secondary deuterium isotope effect, $K_D/K_H = 1.37 \pm 0.03$ by NMR measurements. Comparable values were observed by the less accurate ultraviolet absorption method. The corresponding ratio for addition of 2-mercaptoethanol, determined from the best fit of mean equilibrium constants to the experimental data using eq 2, was $K_D/K_H = 1.25 \pm 0.06$. Examples of experimental observations on mercaptan addition are shown in Figure 1. Table III shows the observed equilibrium constants for addition of water and 2-mercaptoethanol to acetaldehyde and acetaldehyde-*1*-*d*.

Discussion

In previous discussions of the inhibition of proteases by peptide-related aldehydes, it was recognized that these compounds undergo covalent hydration (Westerik and Wolfenden, 1972; Thompson, 1973, 1974; Schultz and Cheerva, 1975). Some authors have also assumed that their extent of hydration may be comparable with the modest extent of hydration of acetaldehyde. It is hardly surprising that hydration of antiproteolytic aldehydes is actually much more extensive, in view of the electron-withdrawing effect of the acylamido function. The special buffering action of hydration, characteristic of aldehydes, must be taken into account when inhibitors with differing functional groups are compared, and may in some cases (see accompanying paper, Lewis and Wolfenden, 1977) have led to serious underestimates of the actual affinity of enzymes for these inhibitors.

Deuterium isotope effects observed in this and earlier studies of reactions of aldehydes with nucleophiles are substantial, and show some specificity for the nucleophile. During this writing, the preliminary results of a study of addition of oxygen nuclephiles to pentanal were published by Hill and Milosevich (1976). The observed isotope effects were $K_D/K_H = 1.39$ for water, and 1.37 for ethanol, closely similar to the present value of 1.37 for water addition to acetaldehyde. An isotope effect of 1.37 has also been observed for nitrogen addition, in the reaction of hydroxylamine with benzaldehyde (do Amaral et al., 1973). In the equilibrium addition of HCN to benzaldehyde, the isotope effect observed by these workers was $K_D/K_H = 1.28$, slightly larger than the present value of 1.25 for addition of 2-mercaptoethanol to acetaldehyde. Results obtained with benzaldehyde may not be strictly comparable with results obtained with aliphatic aldehydes, since changes in hybridization upon adduct formation probably differ in detail. However, it is found in both series that different isotope effects are observed with different nucleophiles.

Effects observed in the present study may be useful for examining structures of enzyme–inhibitor complexes. Thus for an aliphatic aldehyde that is extensively hydrated in solution, deuterium substitution would be expected to result in a substantial increase in observed K_i if the aldehyde were bound unaltered. A smaller increase in observed K_i would be expected if a thiohemiacetal were formed at the active site, and a slight decrease in K_i if the aldehyde were bound as an oxygen adduct (a hydrate or hemiacetal, for example), as discussed in the accompanying paper (Lewis and Wolfenden, 1977).

Halomethyl ketones are widely employed as enzyme inhibitors, but covalent hydration does not seem to have been considered in previous discussions of their reactivity. It seems likely that the inhibition of aminopeptidases by halomethyl ketones, which has been found to be strong but reversible (Birch et al., 1972; Kettner et al., 1974), may involve addition of a nucleophile at the enzyme's active site. The present findings show that 1,3-dibromoacetone, an irreversible inhibitor of proteases (Husain and Lowe, 1968), is strongly hydrated, and this presumably reduces the concentration of the reactive form of the inhibitor in solution. It seems reasonable to suppose that derivatization of papain may proceed through several stages, involving preliminary addition by an enzyme nucleophile to the carbonyl group of the inhibitor, followed by irreversible alkylation. A similar sequence of events has recently been proposed for the reaction of halomethyl ketones with subtilisin BPN (Poulos et al., 1976). Such mechanisms provide a reasonable entropic explanation for the rapid rates that are sometimes observed for reaction between enzymes and halomethyl ketones, but are likely to be difficult to demonstrate except in unusually favorable cases. Where inhibition is reversible, as for aminopeptidases, it should be possible to examine structures of the inhibitory complexes directly in order to determine whether covalent addition has in fact occurred.

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Thiohemiacetal Formation by Inhibitory Aldehydes at the Active Site of Papain†

Charles A. Lewis, Jr.,‡ and Richard Wolfenden*

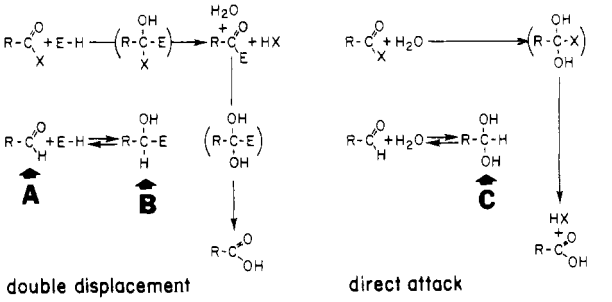
ABSTRACT: Papain is strongly inhibited by aldehydes resembling carboxylic acids released by hydrolysis of specific substrates (Westerik, J. O'C., and Wolfenden, R. (1972), *J. Biol. Chem. 247,* 8195-8197). Inhibitory complexes might involve binding of the aldehyde intact or as a covalent hydrate, or the aldehyde might undergo covalent addition of an active site sulfhydryl group to form a thiohemiacetal derivative. In an attempt to distinguish between these possibilities, benzamidoacetaldehyde-*1-d* has been synthesized, and its properties compared with those of the undeuterated inhibitor. After correction for differences in hydration, the observed effect on inhibition is found to be compatible with formation of a thiohemiacetal. In keeping with this conclusion, benzamidoethanol (a partial analogue of the covalent hydrate) and benzamide, *N*-methylbenzamide and *N*-ethylbenzamide (somewhat similar to the free aldehyde in size and hydrophobic character) are found to exhibit negligible affinity for the active site.

Aldehydes, designed to be capable of forming tetrahedral adducts with nucleophilic residues at the active sites of proteases, are unusually effective reversible inhibitors (Westerik and Wolfenden, 1972; Thompson, 1973, 1974). In the accompanying paper it was shown that peptide-related aldehydes are strongly hydrated, and secondary deuterium isotope effects on equilibrium addition of nucleophiles to aldehydes were compared (Lewis and Wolfender, 1977).

It is of interest to inquire whether aldehydes are indeed bound by enzymes as covalent adducts (Scheme I, case B). Among alternative possibilities, inhibition by an aldehyde hydrate (Scheme I, case C) would accord with a mechanism involving direct attack by water on the peptide bond (Findlater and Orsi, 1973). Aldehydes might also be bound intact, exhibiting unusual affinity in comparison with substrates because of their meager space-filling requirements. This has been suggested as a reasonable basis for the fairly strong inhibition of papain by nitriles (Lowe and Yuthavong, 1971). Recent evidence indicates that several derivatives of cinnamaldehyde are bound without chemical alteration by chymotrypsin (Breaux and Bender, 1975; Gorenstein et al., 1976).

This paper describes experiments designed to discriminate between these possibilities for peptide-related inhibitors of papain, making use of the effect of deuterium substitution on equilibrium addition of nucleophiles to aldehydes, and of analogues with properties resembling other forms in which these inhibitors might be bound.

SCHEME I: Alternative Forms of Bound Aldehyde.[a]



[a] (A) The intact carbonyl compound; (B) a covalent adduct (hemiacetal or thiohemiacetal) formed by addition of a nucleophilic residue at the enzyme's active site; (C) *gem*-diol formed by covalent hydration of the aldehyde. Hydroxyl groups have arbitrarily been drawn in the uncharged state.

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