should be described as a "glassberg" rather than as an "iceberg," see, F. Franks, ref 5, Vol. 2, p 28. Therefore the terms "structure making" and "structure breaking" may be somewhat misleading since they may refer to transitions to structures which are not those characteristic for pure water at 25°.

(29) (a) Alternatively, one could imagine a one-encounter mechanism for the neutral hydrolysis of **1** and **2**. This would involve a cyclic transition state containing three molecules of water; see R. P. Bell, "The Proton in Chemistry," 2nd ed, Chapman and Hall, London, 1973, p 186. Since in this process the proton is transferred to the carbonyl group instead of to water, we feel that this mechanism explains less readily the large changes in $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ induced by adding a few mole per cents of *t*-BuOH; (b) The magnitudes of the OH stretching frequency shifts ($\Delta\nu_{OH}$) for the hydrogen-bonded complexes between phenol as the donor and **1** ($\Delta\nu_{OH}$ = 88 cm^{-1}) or **2** ($\Delta\nu_{OH}$ = 102 cm^{-1}) (in CCl_4) indicate that the substrate molecules are only very weak hydrogen-bond acceptors. The same conclusion holds for *p*-nitrophenyl acetate ($\Delta\nu_{OH}$ = 100 cm^{-1}).

(30) E. M. Arnett and D. R. McKelvey in "Solute-Solvent Interactions," J. F. Coetzee and C. D. Ritchie, Ed., Marcel Dekker, New York, N.Y., 1969, Chapter 6. However, recently Frank has drawn attention to the virtually indistinguishable X-ray scattering curves of H_2O and D_2O at 4° which strongly suggest a closely similar structure for both liquids; see H. S. Frank in ref 5, Vol. 1, p 527.

(31) *Cf.* ref 2, p 258.

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Proton-Exchange Reactions of Acetone and Butanone. Resolution of Steps in Catalysis by Acetoacetate Decarboxylase

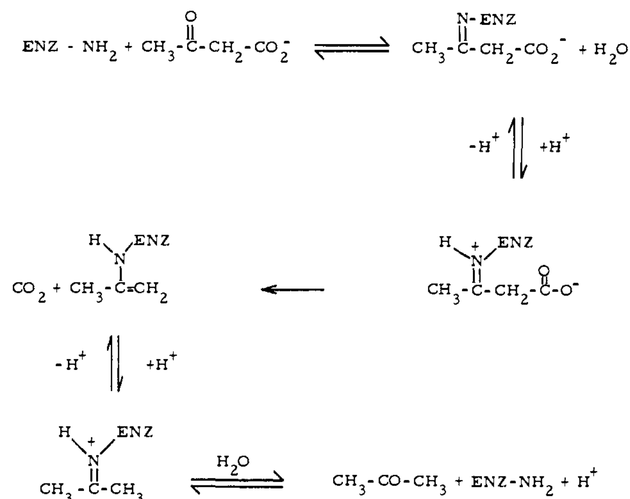
George Hammons,[1a] F. H. Westheimer,*[1a] Kurt Nakaoka,[1b] and Ronald Kluger*[1b,c]

Contribution from the James Bryant Conant Laboratories, Harvard University, Cambridge, Massachusetts 02138, and the Department of Chemistry, University of Chicago, Chicago, Illinois 60637. *Received June 13, 1974*

Abstract: The conversion of acetoacetate to acetone and carbon dioxide, catalyzed by acetoacetate decarboxylase (AAD), involves the enamine of acetone and the enzyme as a compulsory intermediate. The enzyme catalyzes the protonation of this enamine to the corresponding iminium ion. Previous investigations had shown that, consistent with this activity, AAD catalyzes the proton-exchange reactions of acetone. In this study, evidence is presented that AAD will catalyze a stereospecific exchange at the 3 position of butanone, and that the exchange of protons with those on the methyl group of acetone occurs in steps, with the rate constants for exchange of successive protons identical except for statistical factors. The hydrolysis of acetone imine must therefore be rapid compared with proton exchange at carbon.

The decarboxylation of acetoacetate, catalyzed by acetoacetate decarboxylase, occurs by way of imines as intermediates. According to the mechanism proposed for the process, acetoacetate reacts with the ϵ-amino group of an active-site lysine residue to yield an imine of acetoacetate, which then undergoes decarboxylation to form the enamine of acetone; subsequently, protonation of this enamine yields the cation of a second imine, that of acetone.[2-5] A review of the experiments upon which this mechanism is based has recently appeared.[6] The overall mechanism for the decarboxylation is presented in Scheme I.

Scheme I



Tagaki et al.[5] showed that the enzyme will catalyze exchange of the deuterons of acetone-d_6 with the protons of water and similarly exchange the protons of acetone with the deuterons of deuterium oxide. Since these exchange reactions almost certainly proceed by way of the imine of acetone and the enzyme, they are relevant to the enzymic reaction. The present study is concerned with the mechanism of the exchange process.

We have determined by NMR spectroscopy and by mass spectrometry that the AAD catalyzed proton-exchange reactions of acetone occur in a stepwise manner. This leads to the conclusion that hydrolysis of the acetone-derived imine of AAD occurs rapidly compared with tautomerization of the imine to the enamine. Furthermore, the AAD-catalyzed deuteration of butanone at the 3 position is stereospecific, confirming that the proton-exchange reaction itself is enzymic and defining some of the geometric requirements of the active site.

Experimental Section

All reagent grade materials were used as purchased. All nonreagent organic chemicals were purified by distillation or recrystallization. Mr. Jerome V. Connors extracted acetoacetate decarboxylase from *Clostridium acetobutylicum* and purified it by published procedures.[4] The spectroscopic assay method described by Fridovich[7] was used to determine the activity of solutions of the enzyme. All enzyme used in these studies had been crystallized and stored at 5° as a suspension in 50% saturated ammonium sulfate solution.

The deuteration of acetone was followed by NMR spectrometry (Varian HA 100 spectrometer) using 0.15 *M*, pD 5.9, 2-picoline buffer and 0.5 *M* acetone in a manner similar to published proce-

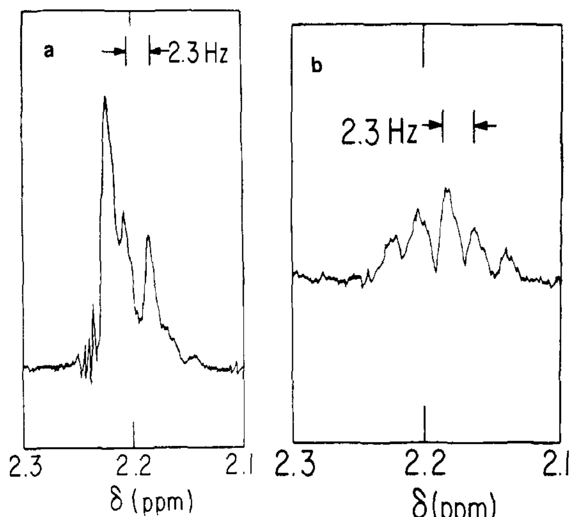


Figure 1. (a) The 100-MHz NMR spectrum of acetone in deuterium oxide after approximately 25% of the proton content of the molecule has been replaced enzymatically with deuterons. (b) The same after about 75% reaction.

dures.[5] The deuteration of butanone was followed in an analogous procedure, using 0.1 M, pD 5.9, phosphate as buffer. Complete high-resolution spectra were taken periodically, and appropriate signals were integrated. Experiments with butanone were performed on a Varian A60 spectrometer. All runs were carried out at 30.0° using NMR tubes which were incubated in a constant-temperature bath. At the end of each run, enzyme activity was assayed; in all cases, enzyme appeared to remain active for the duration of the experiment.

Mass Spectral Procedure. The dedeuteration of acetone-d_6 was followed by mass spectrometry in order that each of the individual species could be looked at separately. The reaction conditions are given in Figures 2a and 2b. All runs were performed at 25°. Aliquots (2 ml) from the 0.5 M or 0.05 M reaction mixture were removed at various times, and the acetone was isolated by the method as outlined by Hamilton.[8] Mass spectra were taken by Dennis Rohrbaugh on an Associated Electrical Industries, Ltd., MS-9. The m/e 58–64 peak heights were measured, and the percentage of each peak height was determined. The acetone-d_6 species was found to disappear in a first-order manner. A plot of the peak percentage of m/e 64 against time gave the rate constant for this disappearance. The other rate constants were determined as described in the Discussion.

Results

Both mass spectra and NMR spectra indicate that acetoacetate decarboxylase catalyzes stepwise exchange in acetone (or deuterioacetone). That is, the reaction proceeds via acetone-d_1, acetone-d_2 . . . acetone-d_6 or the reverse. The experimental results which indicate this are presented in Figures 1 and 2. The protons of the methyl signal of acetone should appear originally as the well-known singlet; then, as monodeuterated species appear, the deuterium–hydrogen coupling should result in the production of a closely spaced triplet (three equal peaks since deuterium has a nuclear-spin quantum number of 1) along with the singlet. As dideuterated species become prevalent, a quintet should appear. These expectations are confirmed experimentally for the deuteration and, in the reverse, for the dedeuteration reaction. Coupling constants (2.3 Hz) are within the range expected from published values.[9] The same stepwise behavior is observed in most nonenzymic cases[10] (see Discussion for the relevant exceptions), although under the conditions of the enzymic experiment, the nonenzymic rate is so slow as to be undetectable.

The rate data for the deuteration of butanone catalyzed by AAD are presented in Figure 2. The plot indicates that all three protons of the 1 position are replaced at a homogeneous rate when the data are plotted as a first-order reaction, while a good plot for deuteration of the 3 position is obtained only when it is assumed that the enzyme is capable of specifically replacing one but not both of the two prochiral protons.[11] The continued deuteration of the 1 position and the final assay which indicated the enzyme retained almost all of its initial activity at the end of the run eliminate the possibility that the cessation of deuteration of the 3 position of butanone after 50% reaction is due to coincidental denaturation of the enzyme. Figure 3 presents NMR data which support the apparent selectivity of the exchange reaction at the 3 position indicated by the kinetic observations. After 90% reaction (that is, the proton signal of the 3 position has decreased to 55% of its original integrated value), the signal of the methyl protons in the 4 position is split by

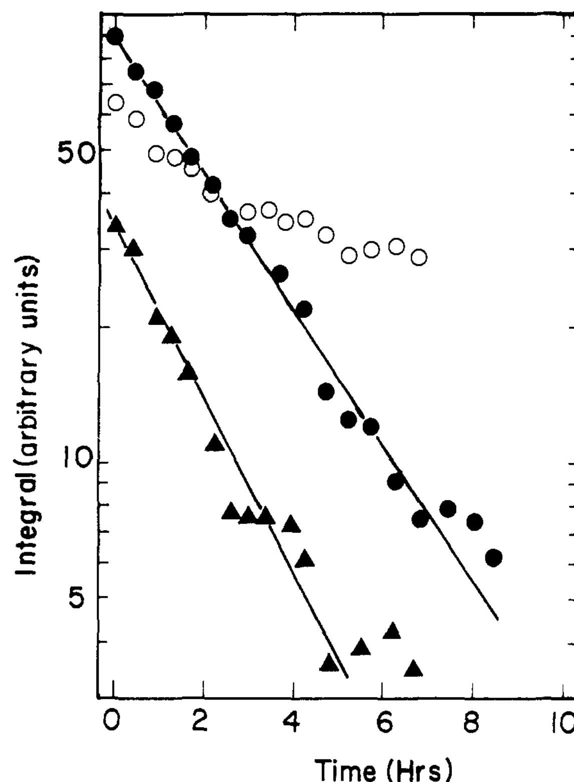


Figure 2. (●) Integrated value of the NMR signals of the 1 position of butanone in deuterium oxide in the presence of approximately 4 × 10^{-6} M acetoacetate decarboxylase at 30.0°. (○) Integrated values of the signal corresponding to the absorption of the protons in the 3 position of butanone under the same conditions. (▲) Data for the 3 position assuming only half of the protons are replaced enzymatically.

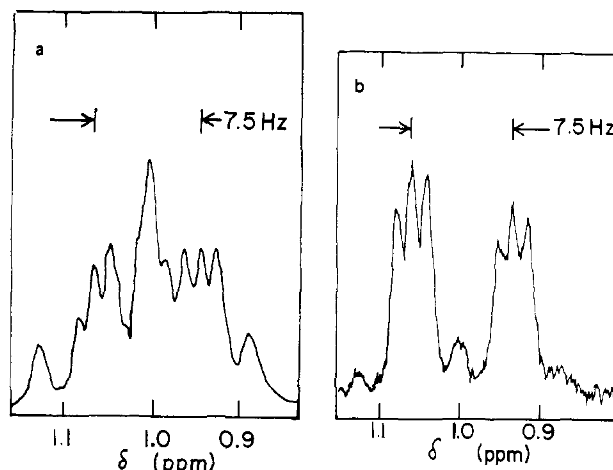


Figure 3. (a) The 60-MHz spectrum of the protons of the 3 position of butanone after 50% of the original signal remains, catalyzed by enzyme; (b) the same except catalyzed by hydroxide.

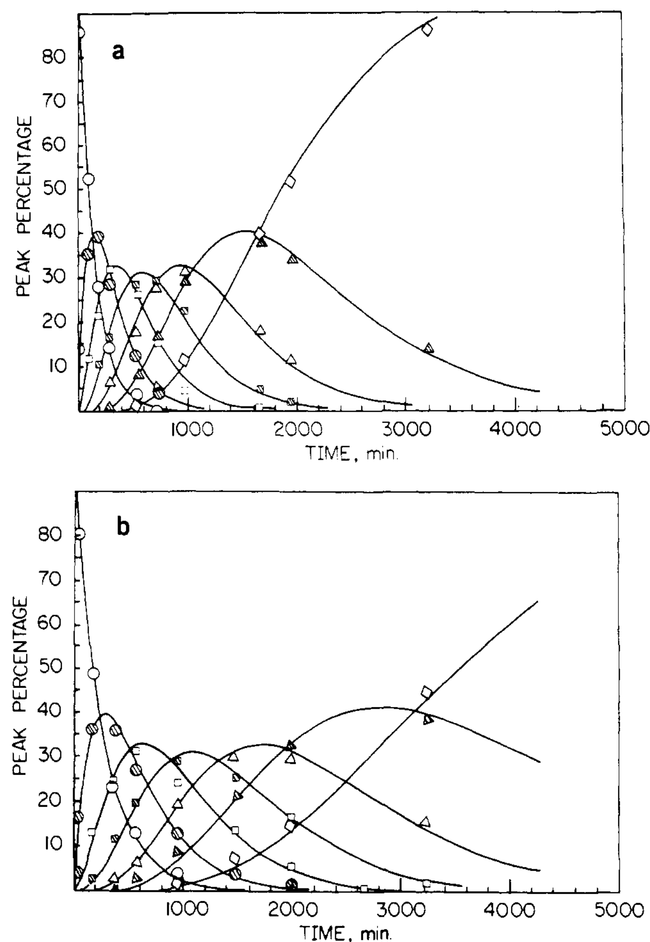


Figure 4. (a) The change with time of the peak height percentages, determined mass spectrometrically, of the various deuterated species of acetone in the dedeuteration of acetone-d_6. The reaction was run using 0.5 M acetone-d_6 in 0.05 M potassium phosphate buffer, pH 5.95, in the presence of 4.9×10^{-6} M (subunits) acetoacetate decarboxylase. (○) acetone-d_6, (⊘) acetone-d_5, (□) acetone-d_4, (⧄) acetone-d_3, (△) acetone-d_2, (▲) acetone-d_1, and (◇) acetone-d_0. The curves are those calculated for a six-step series of consecutive first-order reactions using the appropriate rate constants. (b) Same as Figure 4 except 0.05 M acetone-d_6 was used.

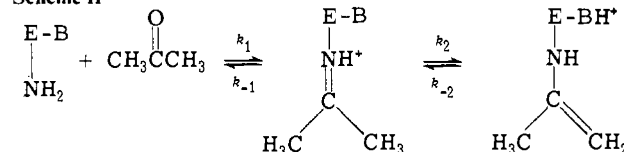
an adjacent methylene group containing one proton and one deuteron (coupling constants and chemical shifts agree with values obtained by others)[9] with a weak triplet remaining due to the residual concentration of species which have not yet incorporated deuterium at the 3 position. The pattern of the signal from the protons at the 4 position eventually becomes that of a methyl group adjacent to a monodeuterated methylene group; no further changes occur in the spectrum after several days. It is this last observation that establishes that only one of the two protons in the 3 position is exchanged enzymically. The data presented in Figure 2 are consistent with and confirm the conclusion since the exchange data give a somewhat better straight line when plotted for exchange of one than for the exchange of both protons. This result requires that the exchange be stereospecific[12] and implicates the involvement of a specific group of the enzyme in the tautomerization reaction. Since replacement of the proton (or deuteron) onto the enamine is stereospecific, by microscopic reversibility,[13] it can be expected that proton removal from the imine is stereospecific and therefore enzymically directed. The involvement of a specific group in the proton abstraction reaction confirms the suggestion of the involvement of a functional group in the active site[3,5,14] in addition to the nucleophilic amino group of the lysine residue which is involved directly in imine formation.[14]

Figure 4 contains the plots of the data obtained in the experiments where the production of the individual species in the dedeuteration of acetone-d_6 was monitored by mass spectrometry. A single rate constant with statistical corrections can accommodate the complicated array of data that result from those measurements. The agreement between experimental data and calculated curves confirms that the exchange reaction occurs in a stepwise manner and gives valuable additional data concerning the rate constant associated with each species. No secondary isotope effect was included in these calculations.

Discussion

The stepwise nature of the exchange indicates that the enzyme-catalyzed proton-exchange reaction of acetone is slow relative to the reaction between acetone and enzyme which forms the imine and the hydrolysis reaction which reproduces enzyme and acetone. Referring to Scheme II, if

Scheme II

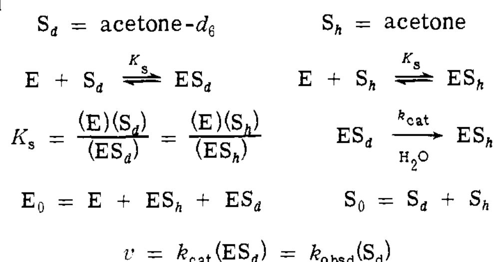


k_{-1} is large compared with k_2, then only stepwise deuteration will be found. If k_{-1} were much smaller than k_2, then the predominant deuterated species that would be observed would be CD_3COCH_3 and acetone-d_6. Since both NMR and mass spectroscopy indicate that single-step deuteration or dedeuteration occurs, k_{-1} must be larger than k_2, and the process corresponding to k_1/k_{-1} can be treated as a preequilibrium.

The involvement of a catalytic group in addition to the lysine residue defines the area adjacent to the imine functionality; the ketone-derived portion of the Schiff base intermediate should have a branch adjacent to the second functional group and a branch remote from that functional group.[15] Once imine formation has occurred only one branch of the molecule is accessible to deuteration (unless the catalytic group is fortuitously equidistant from both positions). Since both branches of butanone are deuterated with approximately the same rate constant (see Figure 2), no large energetic preference obtains for either of the two possible geometric imine isomers. This conclusion is a reasonable consequence of the fact that the enzyme's active site has sufficient space to accommodate the acetoacetate molecule. The stereospecificity of the exchange at the 3 position of butanone indicates that the "ethyl branch" has a specific binding mode in which the C_4 methyl group is best accommodated. This may be the same space as is made available to the carboxylate group of acetoacetate. The report that 2-methylacetoacetate is a rather poor substrate of acetoacetate decarboxylase (k_{cat}/K_m for the methylated derivative is lower by a factor of 22)[16] indicates that the active site does not readily accommodate both the methyl group and the carboxyl function attached to the same carbon atom.

The kinetic expression for the deuteration or dedeuteration process can be treated as a simple Michaelis–Menten system[17] since our observations indicate that proton exchange of the enzymic imine is slow with respect to hydrolysis of the imine. The derivation of the kinetic expression (Chart I) is simplified further by the assumption that deuterated and undeuterated species bind to the enzyme with the same affinity. The expression is valid for the replacement of a single proton or deuteron with statistical corrections or for total exchange. The value of parameters necessary for the calculation of k_{cat} can readily be determined.

Chart I



algebraic manipulation yields: $k_{cat} = k_{obsd}\dfrac{(K_s + S_0)}{E_0}$

The reported value of K_i for the inhibition by acetone[2a] of decarboxylation of acetoacetate is 0.3 *M* which should approximate K_s as defined in the current study. The value of K_s that gives a consistent value for k_{cat} at both concentration levels of the mass-spectral experiments is 0.45 *M*. Since the rate data fit the single parameter curves quite well, it is reasonable to use this particular value for further calculations. The value of k_{cat} obtained assuming one active site per two subunits[18] is 4 sec^{-1}, although other values of K_s lead to a k_{cat}[5] of up to 10 sec^{-1}. This is comparable to values of k_{cat} calculated from data given for a similar process in rabbit muscle aldolase.[5,19]

The mass spectrometric data can be interpreted on the basis of successive reactions, where each step is independent of the others. The first-order rate constant for the first step, the loss of a deuterium atom from acetone-d_6, can easily be determined, and the rest of the data can be accurately represented by assigning to each subsequent step that same rate constant multiplied by the appropriate statistical factor for the particular step. This means that the rate constant assigned to the dedeuteration of acetone-d_5 was $5/6$ of that for the dedeuteration of acetone-d_6; the rate constant for the dedeuteration of acetone-d_4 was $2/3$ of that for acetone-d_6, and so on down to the rate constant for the dedeuteration of acetone-d_1, which was $1/6$ of that for acetone-d_6. Rate equations for successive first-order reactions are known;[20] here we integrated the successive differential rate equations and set up a simple computer program to predict the concentrations of the various partially deuterated species granted the original concentrations of acetone-d_6 and the assumed rate constants. Trials with rate constants for successive steps 10% greater or less than those obtained by the application of statistical factors (as explained above) produced curves that fit the experimental data obviously less well.

A complete analysis of the enzymic system would entail assigning rate constants to each step of the overall process. Insufficient data are as yet available to allow this, and we have not yet designed experiments which would permit gathering the needed information. Hine and his collaborators[21-23] have studied in detail the corresponding nonenzymic reactions, with various ketones, and with both simple amines and bifunctional amines as catalyst. Most primary amines catalyze the stepwise exchange of protons, as in the enzyme case, but a few bifunctional amines catalyze the complete exchange from one of the methyl groups of acetone imine before the other reacts;[23] in these cases, the exchange must be faster than the hydrolysis of the imine. With knowledge of the rate constants for exchange and of the p*K*'s of the catalyst and the imines formed from them, Hine et al. were able to specify the rate constants for protonation and deprotonation.

For the enzymic decarboxylation, however, the needed p*K*'s are not known. The process under consideration can be represented by Scheme II where B represents the basic group on the enzyme that removes the acidic proton of the imine salt, and E represents the enzyme residue. Although some estimate might be made for the p*K*'s of the imine and of the enamine (for protonation on carbon), the p*K* of the basic group, B, or even its chemical nature, is unknown. True, the pH-rate profile for the enzymic decarboxylation is known;[14,24] the pH vs. V_{max}/K_M curve shows a maximum around pH 5.9, controlled by two "apparent" p*K*'s near 6. But in multistep enzymic processes such as this one, the apparent p*K*'s of the essential groups on the enzyme, or the enzyme–substrate complex, correspond to complicated algebraic expressions that contain, in addition to the relevant ionization constants, ratios of rate constants of unknown magnitude.[14]

The value of k_2 could in principle be obtained from the estimated p*K* of the imine and the observed rate constant of 4 sec^{-1} for the loss of a proton from the mixture of imine and imine salt that obtains at pH 5.9. The ratio of k_2/k_{-2} is equal to the quotient K_{SH^+}/K_{BH^+}, where K_{SH^+} is the ionization constant for the protonation of the enamine on carbon, and K_{BH^+} is the ionization constant for the group responsible for catalysis. It is this latter constant which cannot be estimated. Although the investigation of the p*K* of the amino group of the essential lysine residue of the enzyme[25] revealed an additional group near the active site with p*K* about 8, it is by no means clear that this group is the catalytically active one. Under the circumstances, any attempt to estimate k_{-2} seems premature.

One can, however, discuss the value of the rate constant for the reverse reaction, the formation of the enamine. Hine et al.[23] have pointed out that *trans*-2-(dimethylaminomethyl)cyclopentylamine is about three times as effective on a weight basis as is acetoacetate decarboxylase in hydrogen exchange from acetone-d_6. A chemical catalyst may well be superior to an enzyme, especially if the reaction in question is not rate limiting. If (as seems likely) the equilibrium of Scheme II strongly favors the imine salt, then the value of k_{-2} will be large, compared with the rate constant for decarboxylation. There will be no evolutionary pressure to increase the rate of the protonation of the imine formed as an intermediate in decarboxylation so long as the decarboxylation[26] rather than the protonation is rate limiting.

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Model Ligands for Copper Proteins. Proton Magnetic Resonance Study of Acetylhistamine and Acetylhistidine Complexes with Copper(I)

P. A. Temussi* and A. Vitagliano

Contribution from Istituto Chimico, University of Naples, 80134 Naples, Italy. *Received May 6, 1974*

Abstract: The complexes (acetylhistamine)$_2$copper$^+$ and (acetyl-L-histidine)$_2$copper$^+$ have been studied in aqueous solutions by means of proton magnetic resonance spectroscopy. The amide group proved to be unable to complex Cu^+ both alone and in conjunction with the nonspecific imidazole ligand. The Cu^{2+} specific carboxylate group, on the other hand, appears to be a possible site of coordination also for Cu^+ when coupled with the imidazole ligand. These results are interpreted with respect to the problem of the active site of copper proteins with redox activity.

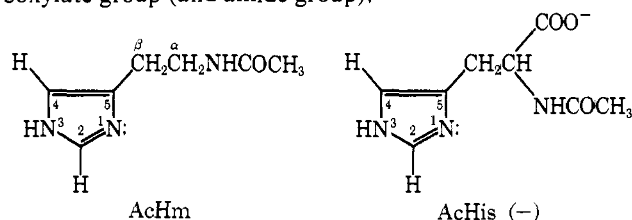
Copper-containing proteins have been the subject of intensive studies[1] during the last few years, both because of their great biological importance (mainly in respiratory chains) and also because of the intrinsic interest posed by the difficult problem of identifying the location of copper in the protein.[2] In fact, this problem is much more difficult than for other metal proteins, *e.g.*, iron heme proteins. In the case of these last proteins, at least four of the six ligands of iron are easily identified upon isolation and characterization of the prosthetic group. On the other hand, in copper proteins, each copper atom may have three to six ligands, all supplied by the residues of the peptide chains,[2] and, accordingly, only direct studies of the proteins can lead to the identification of the ligands. In the case of relatively small proteins, an unequivocal answer to this problem may come from single-crystal X-ray diffraction studies, but it is rather unlikely that any such study will even be attempted for the gigantic molecules of hemocyanins in the near future.

All direct spectroscopic studies[3] have failed to give clear evidence on the structure and composition of the active site of copper proteins (or even for that matter, on the oxidation states of the different copper atoms of a given protein). On account of all these difficulties, indirect studies on model compounds may be of great significance for identifying the probable copper ligands in copper proteins.

In the absence of prosthetic groups, the choice of ligands can be restricted to the side chains of naturally occurring amino acid residues and/or to the peptide chain groups themselves. These ligands can be further subdivided, on the basis of many physicochemical studies, into Cu^{2+} specific, Cu^+ specific, and nonspecific.[4] A very stringent requirement on the composition of the active site of copper proteins involved in oxidation–reduction reactions is that electron transfers occur without ligand displacement. This amounts to saying that the ligands we are looking for must be good for both Cu^{2+} and Cu^+.

In line with these ideas, several researchers in the past have proposed either the sulfhydryl group of cysteine[5] or the imidazole ring of histidine[6] as the linkage of copper to protein in hemocyanins. Other studies, *e.g.*, the effect of photooxidation and histidine reagents on *Murex trunculus* hemocyanin,[7] point to the imidazole ring as a probable ligand rather than the sulfhydryl group. Accordingly we focused our attention on models in which the nitrogen atoms of the imidazole ring could be complexed to Cu^+ or Cu^{2+} together with other ligands very common in protein molecules such as the amide group and/or the carboxylate group which, although specific for Cu^{2+}, might take part in Cu^+ complexation if coupled with a possible Cu^+ ligand.

Acetylhistamine (henceforth referred to as AcHm) was chosen as a model for the case of imidazole ring plus amide group and acetyl-L-histidine (henceforth referred to as AcHis) as a model for the case of imidazole ring plus carboxylate group (and amide group).



Nmr spectroscopy techniques were employed in our study since they are probably unique in giving direct structural information on complexes containing an ion such as Cu^+ which have been generally studied with physicochemical methods that give only very indirect structural information (*e.g.*, potentiometry).

Experimental Section

Materials. Acetonitrile was purchased from C. Erba (Milano, Italy) and purified by distillation over molecular sieves. *N,N*-Dimethylacetamide was obtained from Fluka AG (Buchs, Switzerland) and distilled twice before use. Powdered Cu and $CuSO_4 \cdot 5H_2O$ were from C. Erba. $CuSO_4 \cdot 5D_2O$ was prepared from $CuSO_4 \cdot 5H_2O$ by exhaustive dehydration and subsequent recrystallization from D_2O (99.7%, from C. Erba).