Low-Barrier Hydrogen Bonding in Molecular Complexes Analogous to Histidine and Aspartate in the Catalytic Triad of Serine Proteases†

John B. Tobin, Sean A. Whitt, Constance S. Cassidy, and Perry A. Frey*

Institute for Enzyme Research, The Graduate School, and Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53705

Received February 17, 1995; Revised Manuscript Received April 7, 1995⊗

ABSTRACT: We present spectroscopic evidence for the presence of low-barrier hydrogen bonds (LBHBs) in molecular complexes composed of carboxylic acids and 1-methylimidazole (1-MeIm) dissolved in aprotic organic solvents. A plot of the values of the low-field proton NMR chemical shifts versus the aqueous pK_a of the carboxylic acid exhibits a positive slope for pK_a values below 2.1 and a negative slope for higher pK_a values. The chemical shifts for protons near the maximum in this plot are 18 ppm, similar to that of 18.3 ppm for His^{57}−Asp^{102} in the protonated catalytic triad of chymotrypsin. The chemical shifts for the proton bonded to C2 of 1-MeIm in these complexes also vary with the pK_a of the carboxylic acid and reveal a gradual change from neutral, hydrogen-bonded 1-MeIm in complexes of weaker acids to hydrogen-bonded 1-methylimidazolium ion in complexes of stronger acids. The midpoint chemical shift for the C2 proton corresponds to a carboxylic aqueous pK_a of about 2.1. FTIR spectra of the 1-MeIm−carboxylic acid complexes in $CHCl_3$ indicate that hydrogen bonding is strong and that the complexes are of three types: (a) neutral complexes with the weaker acids (p$K_a \geq 2.2$) in which the antisymmetric carbonyl stretching frequencies are lowered relative to the free acids and the ethyl esters of the same acids; (b) ionic complexes of stronger acids (p$K_a \leq 2.1$) in which the carbonyl stretching frequencies are slightly lower than those for the tetrabutylammonium salts of the same acids; (c) ionic complexes of the same acids (p$K_a \leq 2.1$) coexisting with type b, in which the carbonyl stretching frequencies are intermediate between those for the tetrabutylammonium salts (bond order 1.5) and those of the same acids or their esters (bond order 2.0). The latter complexes appear to incorporate a low-barrier hydrogen bond and are presented as models for the protonated triad of chymotrypsin and other serine proteases. These enzymes have been postulated to utilize a low-barrier hydrogen bond between His^{57} and Asp^{102} to facilitate the abstraction of the β-OH proton from Ser^{195} in the course of catalysis [Frey, P. A., Whitt, S. A., & Tobin, J. B. (1994) *Science (Washington, D.C.) 264,* 1927−1930].

The proton bridging N$^{\delta 1}$ of His^{57} and the β-carboxyl group of Asp^{102} in the active site triad of chymotrypsin resonates at a low field in the NMR spectrum (Robillard & Shulman, 1972; Markley, 1978; Bachovchin, 1985). The low-field proton in the protonated triad has recently been assigned as a low-barrier hydrogen bond (LBHB)[1] (Frey et al., 1994). The evidence presented for this assignment includes the low-field chemical shift of this proton (18.3 ppm) and a deuterium isotope effect on its chemical shift. The protonated triad of the tetrahedral adduct between Ser^{195} of chymotrypsin and *N*-Ac-L-Leu-L-Phe-$COCF_3$ exhibits a chemical shift of 18.7 ppm, and the pK_a of the triad in this complex is greater than 10.5 (Liang & Abeles, 1987). These facts have led to the postulation of a new mechanism for the action of chymotrypsin, in which the strength of the LBHB facilitates the formation of the tetrahedral adduct according to Scheme 1 (Frey et al., 1994).

A prerequisite for LBHB formation is that the pK_as of the conjugate acids of the interacting groups must be matched within their microenvironment (Hibbert & Emsley, 1992; Frey et al., 1994; Cleland & Kreevoy, 1994; Gilli et al., 1994; Gerlt & Gassman, 1993). The interaction of His^{57} and Asp^{102} can in principle lead to low-barrier hydrogen bonding in microenvironments in which the acidities of the imidazolium ring and the carboxylic acid group are similar; such a microenvironment can be provided by an enzymatic active site (Frey et al., 1994). The interaction of these groups in an aqueous environment could not lead to an LBHB because their pK_as are not matched in water and LBHBs cannot be formed in water.

The nature of the interaction between His^{57} and Asp^{102} in the protonated triad has not been explored in chemical models, although *cis*-urocanic acid dissolved in DMSO has been presented as a first approximation to such a model (Frey et al., 1994). We have now applied NMR and FTIR spectroscopy to study a series of intermolecular complexes formed between carboxylic acids and 1-methylimidazole (1-

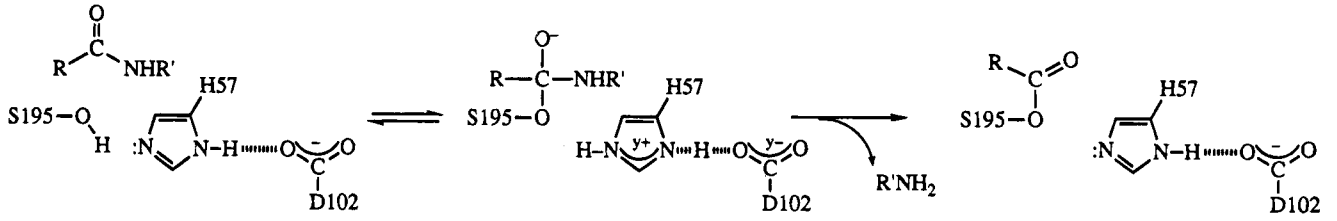
† Supported by Grants DK 28607 from the National Institute of Diabetes and Digestive and Kidney Diseases and GM 51806 from the National Institute of General Medical Sciences. This study made use of the National Magnetic Resonance Facility at Madison, which is supported by NIH Grant RR02301 from the Biomedical Research Technology Program, National Center for Research Resources. Equipment in the facility was purchased with funds from the University of Wisconsin, the NSF Biological Instrumentation Program (Grant DMB-8415048), the NIH Biomedical Research Technology Program (Grant RR02301), the NIH Shared Instrumentation Program (Grant RR02781), and the U.S. Department of Agriculture.

⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1995.

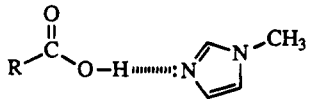
[1] Abbreviations: LBHB, low-barrier hydrogen bond; NMR, nuclear magnetic resonance; FTIR, Fourier transform infrared; 1-MeIm, 1-methylimidazole; DMSO, dimethyl sulfoxide.

0006-2960/95/0434-6919$09.00/0

Scheme 1



MeIm) of the type illustrated below in aprotic organic solvents:



where R = CF_3 (**1**), $CHCl_2$ (**2**), $C(CH_3)Cl_2$ (**3**), CH_2Cl (**4**), CH_3OCH_2 (**5**), $PhCH_2$ (**6**), and CH_3 (**7**).

We were interested in learning whether these complexes would exhibit very low-field signals for a strongly hydrogen-bonding acidic proton. We chose 1-MeIm instead of imidazole in order to minimize hydrogen-bonding possibilities in the solutions and to focus attention on the proton bridging the carboxylic acid and imidazole nitrogen. The results of our NMR and FTIR experiments support the presence of LBHBs in these complexes.

EXPERIMENTAL PROCEDURES

Materials. All chemicals and solvents were obtained from commercial suppliers and repurified by either recrystallization or redistillation. Solids were thoroughly dried in a vacuum desiccator. Solvents were dried over molecular sieves, dried by refluxing over P_2O_5 followed by distillation, or purchased from Aldrich Chemical Co. as anhydrous solvents in sealed vials, all of which gave equivalent results. The purity of all chemicals and the absence of water were verified by their relevant physical and spectroscopic properties.

Spectroscopy. For NMR and FTIR studies, each of seven carboxylic acids (trifluoroacetic, dichloroacetic, 2,2-dichloropropanoic, chloroacetic, methoxyacetic, phenylacetic, or acetic acid) was mixed in 1:1 stoichiometry with 1-MeIm and dissolved in $CDCl_3$, CD_2Cl_2, or C_6D_6 at concentrations of 50 mM to 0.5 M. The solutions were prepared in a drybox and carefully protected from atmospheric moisture throughout all spectroscopic experiments.

The stronger carboxylic acids (aqueous $pK_a < 2$, complexes **1**–**2**) form very strong complexes with 1-MeIm, and the observed low-field proton chemical shift δ_{obs} drifts upfield with increasing concentration. The chemical shift δ_{BHA} for the 1:1 complex was determined by a linear extrapolation of δ_{obs} to zero complex concentration. Complexes formed between 1:1 mixtures of weaker acids ($pK_a > 3$, acids **5**–**7**) and 1-MeIm are much weaker and exhibit increasingly downfield chemical shifts at increasing concentrations. The concentration of the 1:1 complex ([BHA]) is governed by a dissociation constant, $K_d = [HA][B]/[BHA] = [HA]^2/[BHA]$, and the expression for the observed chemical shift is given by the equation $\delta_{obs} = \delta_{BHA} + (\delta_{HA} - \delta_{BHA})[(K_d^2 + 4K_d[HA]_o)^{1/2} - K_d]/(2[HA]_o)$, where δ_{HA} and δ_{BHA} are the chemical shifts of the acidic proton in the acid and its complexes with 1-MeIm. Values for δ_{BHA} were obtained by fitting data to this equation. Increasing the ratio 1-MeIm:

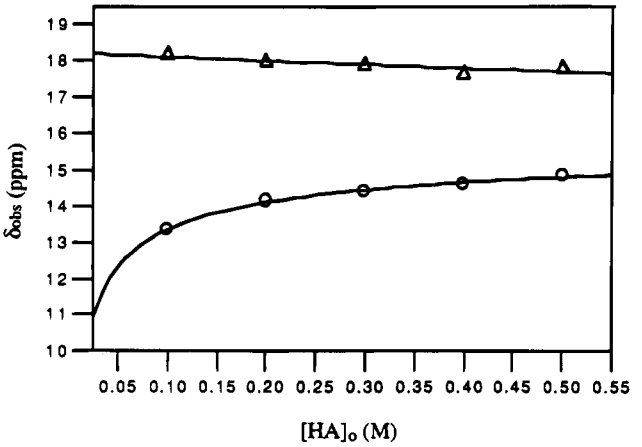


FIGURE 1: Concentration dependence of chemical shifts (δ_{obs}) for the acidic proton in equimolar mixtures of carboxylic acids and 1-MeIm in $CDCl_3$. Symbols: triangles, a typical plot of δ_{obs} against concentration for the strong complex of 1:1 dichloroacetic acid:1-MeIm; open circles, a typical plot of δ_{obs} against concentration for the weak complex of 1:1 acetic acid:1-MeIm. Values of δ_{BHA} were determined from these plots as described in Experimental Procedures.

phenylacetic acid to 2:1 did not change the value of δ_{BHA}. The values for limiting chemical shifts were well determined from data obtained near saturation; however, δ_{HA} and K_d were poorly determined under these conditions. We were most interested in δ_{BHA} and chose conditions for evaluating them as accurately as possible. Estimation of K_d produced values in the range of 0.1 M. The values of δ_{obs} for complexes formed by acids **3** and **4** showed no concentration dependence and were accepted as δ_{BHA} for those complexes.

RESULTS

NMR Properties of Carboxylic Acid-1-MeIm Complexes. One-to-one stoichiometric mixtures of 1-MeIm with carboxylic acids dissolved in aprotic solvents such as $CDCl_3$, CD_2Cl_2, and C_6D_6 exhibit low-field signals for the acidic, hydrogen-bonded proton linking the carboxylic acid and imidazole groups. The chemical shift (δ_{obs}) of the acidic proton varies with the concentration of the complex, the acidity of the carboxylic acid, and to a lesser extent the solvent. Plots of chemical shift versus acid concentration exhibit three types of behavior, two of which are illustrated in Figure 1. Acids exhibiting aqueous pK_as less than 2 (acids **1**–**2**) form very strong complexes with 1-MeIm and exhibit their maximally downfield signal in the most dilute solutions. The slight upfield drift in δ_{obs} with increasing concentration is presumably due to the formation of higher aggregates, an effect also observed in a few pyridine–trifluoroacetic acid complexes (Dega-Szafran & Kuzendorf, 1979). Therefore, the value of δ_{BHA} for the 1:1 complex is obtained by a linear extrapolation of δ_{obs} to zero complex concentration. Complexes formed between weaker acids ($pK_a > 3$, acids **5**–**7**)

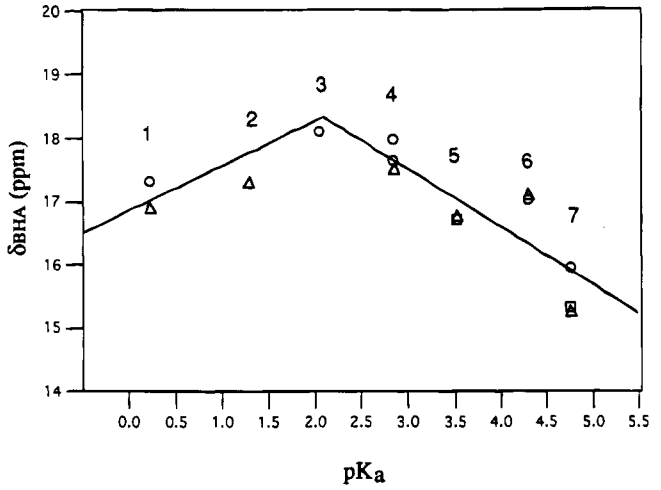


FIGURE 2: Dependence of the chemical shifts for the acidic protons in carboxylic acid−1-MeIm complexes on the aqueous pK_as of the carboxylic acids. Limiting values of δ_{BHA} for 1:1 complexes of carboxylic acids with 1-MeIm are plotted against aqueous pK_as of the carboxylic acids. Symbols: $CDCl_3$ (circle), CD_2Cl_2 (triangle), and C_6D_6 (square). The intersection point for the two linear fits occurs at pK_a = 2.05. The carboxylic acids are **1**, F_3CCOOH; **2**, $Cl_2CHCOOH$; **3**, CH_3CCl_2COOH; **4**, $ClCH_2COOH$, **5**, CH_3OCH_2-$COOH$; **6**, $PhCH_2COOH$; and **7**, CH_3COOH.

and 1-MeIm are much weaker and exhibit increasingly downfield chemical shifts at higher concentrations (lower curve in Figure 1). In these cases, the values of δ_{BHA} are obtained by fitting data to a binding equation as described in Experimental Procedures. Data obtained at higher concentrations give good values for δ_{BHA}. The dissociation constants are not well determined under these conditions, but the estimated values of about 0.1 M confirm the weakness of complexation by weaker acids. The chemical shifts for complexes formed by acids **3** and **4** show little or no concentration dependence and give values of δ_{BHA} for 1:1 complexes at concentrations ≤0.5 M.

The values for δ_{BHA} in various solvents are plotted in Figure 2 against the acidities of the carboxylic acids, which are expressed as their aqueous pK_as (numerical values appear in the supplementary material). The chemical shifts are further downfield than those for imidazole or the 1-methylimidazolium ion (7−12 ppm depending on solvent). Values of δ_{HA} for the same acids dissolved in $CDCl_3$, in which they presumably form hydrogen-bonded carboxylic acid dimers, are further upfield. They range from 11.2 to 11.9 ppm at 0.5 M acid and from 9.9 to 11.4 ppm at 0.2 M acid.[2]

The plot of δ_{BHA} versus pK_a in Figure 2 is biphasic and is depicted as a simultaneous fit to two straight lines of positive and negative slopes intersecting at pK_a = 2.1 and 18 ppm. The maximum in this plot corresponds to the strongest hydrogen bonding in this series under the experimental conditions. In $CDCl_3$ the proton seems to be equally shared between 1-MeIm and a carboxylic acid with an aqueous pK_a of 2.1. The difference of 4.9 between the aqueous pK_a of *N*-methylimidazolium (7.06; Nozaki et al., 1957) and a carboxylic acid of pK_a 2.1 corresponds to matched acidities in chloroform. This is because the pK_a of the carboxylic acid will be elevated by about 5 pK_a units in an aprotic medium, whereas the pK_as of positively charged acids such as the *N*-methylimidazolium ion will be similar to their values in water (Bell, 1959).

[2] Higher oligomers of hydrogen-bonded carboxylic acids might also be possible in addition to the dimers. Such oligomers would also be linked by two hydrogen bonds per carboxylic acid, so that all of the arguments advanced in this paper with respect to the relative strengths of the hydrogen bonds would apply to them in the same way as to the carboxylic acid dimers.

Table 1: Values of Chemical Shifts for the C2 Proton of 1-MeIm in Hydrogen-Bonded Complexes with Acids of Varying Acidities

acid	pK_a	δ_{2H} (ppm)[a]
p-$MePhSO_3H$	−3	8.93
F_3CCO_2H	0.23	8.97
Cl_2CHCO_2H	1.29	8.48
$CH_3CCl_2CO_2H$	2.06	8.39
$ClCH_2CO_2H$	2.86	8.02
$CH_3OCH_2CO_2H$	3.53	7.79
$PhCH_2CO_2H$	4.31	7.59
CH_3CO_2H	4.76	7.56
none (*N*-MeIm)[b]		7.42

[a] Values of the chemical shift for the C2-proton of 1-MeIm mixed in 1:1 stoichiometry with the indicated acid at 0.2 to 0.5 M in $CDCl_3$ at 25 °C. [b] 1-MeIm dissolved in $CDCl_3$.

In 1-MeIm complexes of the more acidic carboxylic acids (pK_a < 2) the proton is mainly associated with the nitrogen in the imidazole ring, and the complexes are ionic. In complexes of weaker acids (pK_a > 3) the proton is mainly bonded to the carboxylic acid, and the complexes are essentially neutral. The chemical shift of the C2 proton in the imidazole ring (δ_{2H}) is a probe of the positive charge state for the imidazole ring. The values for δ_{2H} for the 1-MeIm complexes are given in Table 1 and show a gradual transition from the value for free 1-MeIm (δ = 7.42 ppm) to that for the 1-methylimidazolium ion (δ = 8.93 ppm). The midpoint δ_{2H} in this series is 8.2 ppm and corresponds to an aqueous carboxylic pK_a value of 2, which coincides with the maximum in Figure 2. The drift in chemical shift for the 2-proton in this series of complexes is remarkably gradual. By this criterion, the difference in positive charge on 1-MeIm is nominal between its complex with chloroacetic acid (δ = 8.02 ppm) and 2,2-dichloropropionic acid (δ = 8.39), which appear near the maximum in Figure 2. This indicates a gradual accrual of positive charge on the neutral complexes with increasing acidity of the carboxylic components. On the acidic side of the maximum in Figure 2 the complexes become ionic, but the positive charge on the imidazolium component is not yet fully formed owing to strong hydrogen bonding to the carboxylate. The positive charge in the methylimidazolium ring increases further with the acidity of the acid.

FTIR Spectra of 1-MeIm−Carboxylic Acid Complexes. To gain further information about the nature of hydrogen bonding in these complexes, their FTIR spectra were obtained in $CHCl_3$. The FTIR spectrum for 2,2-dichloropropionic acid and that of its complex with 1-MeIm in $CHCl_3$ are shown in Figure 3. The broad band marked A in Figure 3A corresponds to O−H stretching in the free acid, and bands for the 1-MeIm complex marked B and C in Figure 3A are typical of O−H stretching frequencies that have been assigned to strong hydrogen bonds in other molecules (Barrow, 1956; Hadzi, 1965; Johnson & Rumon, 1965; Lindeman & Zundel,1972, 1976; Hadzi & Klibarov, 1966). The frequencies corresponding to the antisymmetric C=O stretching modes ($\nu_{C=O}$) are of particular interest. This well-defined band appears at 1734 cm^{-1} for the free acid (Figure

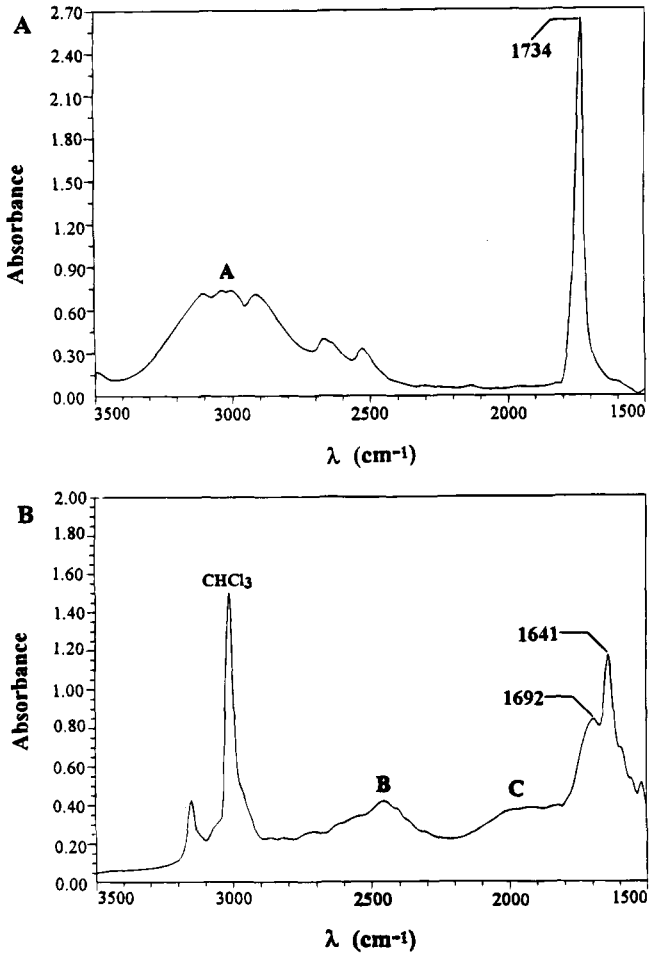


FIGURE 3: FTIR spectra of 2,2-dichloropropionic acid and its complex with 1-MeIm. (Part A) FTIR spectrum for 2,2-dichloropropionic acid in $CHCl_3$. The band for antisymmetric C=O stretching is well defined at 1734 cm^{-1}. The band marked A is the A-band for the O−H stretching mode. (Part B) FTIR spectrum for a 1:1 mixture of 2,2-dichloropropionic acid and 1-MeIm in $CHCl_3$. The antisymmetric C=O stretching region reveals two bands at 1692 and 1641 cm^{-1} corresponding to at least two different complexes. The species corresponding to 1641 cm^{-1} is assigned as a conventional ionic complex, and that corresponding to 1692 cm^{-1} is assigned to an LBHB-bonded complex.

3A). However, in the case of the complex with 1-MeIm the C=O region is broadened and attenuated and appears as at least two bands. Spectral deconvolution resolves the C=O region for this complex into three bands, one at 1641 cm^{-1}, a second at 1692 cm^{-1}, and a third very small band at 1727 cm^{-1} corresponding to the free acid. Species corresponding to the first two of these are also displayed by the ionic complexes formed from dichloroacetic acid and trifluoroacetic acid.

Interpretation of the FTIR data on antisymmetric C=O stretching modes for the 1-MeIm complexes is aided by consideration of the corresponding frequencies for the methyl esters, the free acids, and tetrabutylammonium salts of the same acids, which are shown in plots of frequency versus aqueous pK_a of the acid in Figure 4. The upper line correlates the frequencies for the methyl esters, in which the C=O bond order is 2.0 and hydrogen bonding is absent. The lower line correlates the frequencies for the tetrabutylammonium salts of the same acids, in which the C=O bond order is 1.5 and hydrogen bonding is absent. Both lines exhibit negative slopes owing to the effects of electron-

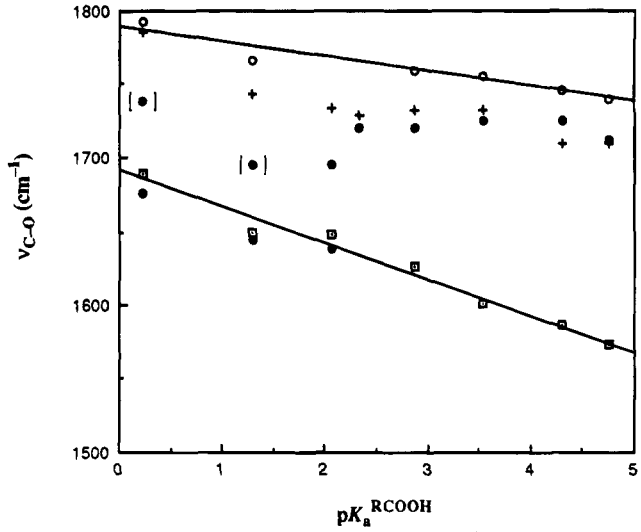
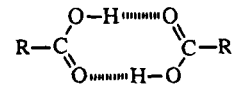


FIGURE 4: Dependence of antisymmetric stretching frequencies for 1:1 complexes of carboxylic acids with 1-MeIm on the acidity of the carboxylic acid. Shown here are the antisymmetric C=O stretching frequencies for various forms of eight carboxylic acids in apolar solvents plotted against the aqueous pK_as for the acids. The acids are, in order of increasing pK_a, F_3CCOOH, $Cl_2CHCOOH$, CH_3CCl_2COOH, $BrCH_2(Br)CH_2COOH$, $ClCH_2CH_2COOH$, CH_3OCH_2-$COOH$, $PhCH_2COOH$, and CH_3COOH. Symbols: open circles, methyl esters (neat); plusses, 0.2 M free acids in $CHCl_3$; closed circles, 0.2 M 1:1 complexes of the acids with 1-MeIm dissolved in $CHCl_3$; open squares, 0.2 M Bu_4N^+ salts of the acids dissolved in $CHCl_3$. The concentration of 0.2 M was chosen for these experiments because the NMR data used to determine values of δ_{BHA} in Figures 1 and 2 indicated that complexation of these acids with 1-MeIm is largely complete at 0.2 M. The lines through the open circles and open squares are correlation lines resulting from linear fits of the frequencies. The slopes of the lines represent inductive effects on the C=O bonds. The scatter about the lines represents real deviations from perfect correlations. The deviations result from secondary effects of the different substituents on the carbonyl groups. The uncertainties in the values of the frequencies are ±2 cm^{-1}.

withdrawing substituents such as F, Cl, and Br, which destabilize the charge-separated resonance forms of the carbonyl groups (C=O ↔ $^+C-O^-$). Inductive electron withdrawal lowers the pK_a and strengthens the carbon−oxygen bond. The deviations of the free acids and the complexes with 1-MeIm or *N*-methylimidazolium ion from these correlation lines give information about hydrogen bonding.

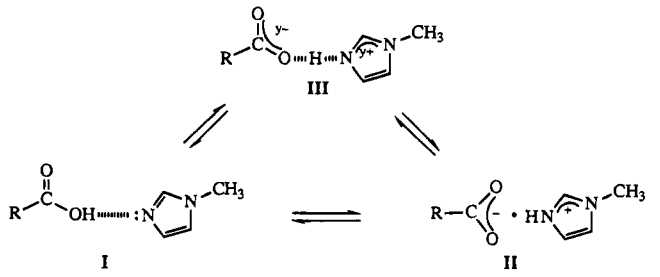
In organic solvents, carboxylic acids form dimeric, hydrogen-bonded complexes, which exhibit frequencies $\nu_{C=O}$



that are lowered relative to those for the esters. The frequencies increase with increasing acid strength, as for the esters and salts. However, there is a significant deviation from linearity owing to stronger hydrogen bonding in the acids of intermediate acidities. The deviations result from secondary structural effects exerted by the different substituents on the carbonyl groups.

The values of $\nu_{C=O}$ for the 1-MeIm complexes with the the weaker acids (aqueous $2 < pK_a < 4$) are lower than those for the free acids and much lower than for the esters. They are either independent of pK_a or exhibit a slight positive

Scheme 2



slope. These frequencies clearly show that single hydrogen bonds linking carboxylic acids and 1-MeIm are stronger than the dual hydrogen bonds linking carboxylic acid dimers. They also reveal a strengthening of hydrogen bonding in the neutral carboxylic acid−1-MeIm complexes as the acid strength increases toward the transition point near pK_a 2.1.

At the point of equal acidities for carboxylic acid and imidazolium species, the ionic complex between 1-MeIm and 2,2-dichloropropionic acid appears as two species in Figure 4 corresponding to the two bands in Figure 3B. One has a slightly lower frequency than that of the corresponding tetrabutylammonium salt and corresponds to a C=O bond order of slightly less than 1.5. The other frequency is *intermediate* between those of the salt and the ester and corresponds to a C=O bond order intermediate between 1.5 and 2. This latter frequency is lower than that for the hydrogen-bonded neutral complex and higher than that for the salt. It is in the range expected for LBHBs that incorporate partial covalent character in the hydrogen bond. Covalency associated with an LBHB can be expected to raise the bond order of the carboxylate ion toward that of the ester. Similar bands for complexes of dichloroacetic acid and trifluoroacetic acid are observed; however, the points corresponding to the intermediate frequencies for the LBHB species are bracketed in Figure 4 to indicate that these are minor components in the C=O region. The mismatch in acidities for these acids and the 1-methylimidazolium ion in $CHCl_3$ may be the reason that the intermediate bands are minor components of the C=O region.

DISCUSSION

The simplest interpretation of the present results is that the nature of the hydrogen bonding in complexes of carboxylic acids with 1-MeIm varies with the acidity of the carboxylic acid and corresponds to at least three types of complexes illustrated in Scheme 2. These are conventional hydrogen-bonded complexes between a carboxylic acid and 1-MeIm (**I**), ion-paired complexes of carboxylate ion with 1-methylimidazolium ion (**II**), and strongly hydrogen-bonded complexes such as the low-barrier hydrogen-bonded complex **III**, in which $0.5 \leq y \leq 1$. Under the conditions of the present experiments, the NMR data probably represent the presence of more than one type of complex between the carboxylic acid and 1-MeIm that are in equilibrium on the NMR time scale. The weaker acids clearly form complexes of type **I**, and the stronger acids form ionic complexes of types **II** and **III**.

The intermediate values of the chemical shifts for the C2 proton of 1-MeIm in Table I might be explained by complexes of types **I** and **II** that are in equilibrium and in fast chemical exchange. However, chemical exchange between **I** and **II** alone would not account for the behavior of the chemical shift of the acidic protons in Figure 2 because the observed values attain a maximum that is higher than that for complexes corresponding to either **I** or **II**.[3] Therefore, at least one additional species such as the low-barrier hydrogen-bonded species **III** must be invoked to explain Figure 2. Species **III** might be represented by the chemical shift of 18 ppm if it dominates the equilibrium or it might contribute significantly if it does not dominate the equilibrium. In the latter case, the value of the chemical shift for **III** must be higher than 18 ppm. We note that the chemical shift for the corresponding proton in the tetrahedral adduct of chymotrypsin is reported to be 18.7 ppm (Liang & Abeles, 1987), so that the 18 ppm estimated from Figure 2 for this interaction probably represents a mixture of species, one of which may be complex **III**.

The presence of low-barrier hydrogen-bonded species in these complexes is most clearly shown by the FTIR data in Figures 3 and 4. Unlike NMR chemical shifts, the stretching frequencies represent discrete complexes and not mixtures in chemical exchange. The complexes exhibiting values of antisymmetric C=O stretching frequencies intermediate between those for the neutral and purely ionic complexes **I** and **II** correspond to species in which the C=O bond order is between 1.5 and 2. Low-barrier hydrogen-bonded species **III** can account for intermediate C=O bond order. The presence of broad bands near 2500 and 2000 cm^{-1} in Figure 3 corresponding to B- and C-type O−H stretching bands is consistent with the presence of strong hydrogen bonds.

Our results indicate that strongly hydrogen-bonded complexes are formed when the carboxylic acidity corresponds to a pK_a of 2.06 (CH_3CCl_2COOH). Given that the pK_a of the β-carboxyl group in aspartate is normally 4.5, the microenvironment of Asp^{102} would have to depress its β-carboxylic acidity for it to form an LBHB with His^{57}. Hydrogen bond donation to Asp^{102} would increase its acidity. Therefore, the available spectroscopic and structural facts are compatible with strong hydrogen bonding in the protonated triad.

The present results should be compared with analogous results for complexes of pyridines of varying basicity with trifluoroacetic acid (Dega-Szafran & Szafran, 1994; Dega-Szafran & Kuzendorf, 1979; Dega-Szafran & Dulewicz, 1981, 1983; Bareczynski et al., 1985) and of pyridine with various stoichiometric ratios of acetic acid (Golubev et al., 1994). Studies of pyridine complexes are analogous to those presented here, except that the basic strength of pyridines was varied and the carboxylic component held constant. Complexes of 1-MeIm with carboxylic acids should be to some degree analogous to interactions of His^{57} and Asp^{102}. FTIR data reported on trypsin and model hydrogen bonding networks are consistent with strong hydrogen-bonding in the triad (Zundel, 1992; Wellner & Zundel, 1994).

The spectroscopic results presented here support the assignment of LBHBs in complexes of carboxylic acids with 1-MeIm when acidities are matched. However, they do not reveal the strengths of these hydrogen bonds. This is a

[3] The lowest values of δ_{BHA} in Figure 2 (17 ppm) may correspond to complex **II** but do not represent the 1-methylimidazolium ion itself because our measurement of the chemical shift for the acidic proton in 1-methylimidazolium *p*-toluenesulfonate in $CDCl_3$ shows it to be only 7 ppm.

complex subject that requires further research employing different methods. It is clear that the LBHB formed between 2,2-dichloropropionic acid and 1-MeIm is approximately as strong as the purely ionic complex in free energy terms. Because the entropic barrier to the formation of the LBHB complex **III** is likely to be higher than that for the ionic complex **II**, it may be that the enthalpic stabilization in complex **III** is higher than that in complex **II**.

Schowen and co-workers have suggested that two strong hydrogen bonds contribute to the stabilization of the transition state for serine protease reactions (Stein et al., 1983; Schowen, 1988). They based their suggestion on the observation of small deuterium kinetic isotope effects in the D_2O/H_2O mixtures used for proton inventory studies. The mechanism in Scheme 1 specifies the location of an LBHB in an intermediate and implies that it stabilizes the preceding transition state. Specific evidence for this LBHB is its low-field chemical shift and the deuterium isotope effect on its chemical shift (Frey et al., 1994). J. L. Markley has recently added to this evidence by measuring a low value of the fractionation factor for the LBHB in the protonated triad of chymotrypsinogen.[4]

SUPPLEMENTARY MATERIAL AVAILABLE

Table 2 giving chemical shifts of protons in complexes of carboxylic acids with *N*-methylimidazole and Table 3 showing infrared stretching frequencies for carboxylic acids, tetrabutylammonium carboxylate salts, and 1:1 complexes of carboxylic acids with *N*-methylimidazole in chloroform solution (3 pages). Ordering information is given on any current masthead page.

REFERENCES

Bachovchin, W. W. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7948–7951.

Barezynski, P., Dega-Szafran, Z., & Szafran, M. (1985) *J. Chem. Soc., Perkin Trans. 2*, 765–771.

Barrow, G. M. (1956) *J. Am. Chem. Soc. 78*, 5802–5806.

Bell, R. P. (1959) *The Proton in Chemistry*, pp 36–61, Cornell University Press, Ithaca, NY.

Cleland, W. W. & Kreevoy, M. M. (1994) *Science (Washington, D.C.) 264*, 1887–1890.

Dega-Szafran, Z., & Kuzendorf, J. (1979) *Pol. J. Chem. 53*, 623–630.

Dega-Szafran, Z., & Dulewicz, E. (1981) *Org. Magn. Reson. 16*, 241–245.

Dega-Szafran, Z., & Dulewicz, E. (1983) *J. Chem. Soc., Perkin Trans. 2*, 345–351.

Dega-Szafran, Z., & Szafran, M. (1994) *Heterocycles 37*, 627–659.

Frey, P. A., Whitt, S. A., & Tobin, J. B. (1994) *Science (Washington, D.C.) 264*, 1927–1930.

Gerlt, J. A., & Gassman, P. A. (1993) *Biochemistry 32*, 11943–11952.

Gilli, P., Bertalasi, Ferretti, & Gilli, G. (1994) *J. Am.Chem. Soc. 116*, 909–915.

Golubev, N. S., Smirnov, S. N., Gindin, V. A., Denisov, G. S., Benedict, H., & Limbach, H.-H. (1994) *J. Am. Chem. Soc. 116*, 12055–12056.

Hadzi, D. (1965) *Pure Appl. Chem. 11*, 435–440.

Hadzi, D., & Klibarov, N. (1966) *J. Chem. Soc. A*, 439–442.

Hibbert, F., & Emsley, J. (1990) *Adv. Phys. Org. Chem. 26*, 255–379.

Johnson, S. L., & Rumon, K. A. (1965) *J. Phys. Chem. 69*, 74–81.

Kreevoy, M. M., & Liang, T. M. (1980) *J. Am. Chem. Soc. 102*, 3315–3322.

Liang, T.-C., & Abeles, R. H. (1987) *Biochemistry 26*, 7603–7608.

Lindeman, R., & Zundel, G. (1972) *J. Chem. Soc., Faraday 2 68*, 979–985.

Lindeman, R., & Zundel, G. (1976) *J. Chem. Soc., Faraday 2 73*, 788–793.

Markley, J. L. (1978) *Biochemistry 17*, 4646–4656.

Nozaki, Y., Gurd, F. R. N., Chen, R. F., & Edsall, J. T. (1957) *J. Am. Chem. Soc. 79*, 2123–2129.

Robillard, G., & Shulman, R. G. (1972) *J. Mol. Biol. 71*, 507–512.

Schowen, R. L. (1988) *Mechanistic Principles of Enzyme Activity* (Liebman, J. F., & Greenberg, A., Eds.) p 119, VCH Publishers, New York.

Stein, R. L., Elrod, J. P., & Schowen, R. L. (1983) *J. Am. Chem. Soc. 105*, 2446–2452.

Wellner, N., & Zundel, G. (1994) *J. Mol. Struct. 317*, 249–254.

Zundel, G. (1992) *Trends Phys. Chem. 3*, 129–154.

BI9503615

[4] Personal communication from J. L. Markley. In a personal communication to the authors, C. J. Halkides has also reported a low fractionation factor for the protonated triad in subtilisin. Low fractionation factors are displayed by LBHBs (Kreevoy & Liang, 1980).