A Simple Method for Determining the Relative Strengths of Normal and Low-Barrier Hydrogen Bonds in Solution: Implications to Enzyme Catalysis

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Abstract: A simple method has been developed to study the relative strengths of normal and low-barrier hydrogen bonds in solution using the maleic/fumaric and mesaconic/citraconic acid equilibria. Isomerization of maleic and fumaric acids was catalyzed by thiourea and isomerization of mesaconic and citraconic acids was catalyzed by the thiophenyl radical, with equilibrium isomeric ratios determined by HPLC. The monoanion of mesaconic and citraconic acid was found to greatly favor the cis isomer in aprotic solvents under conditions in which the cis isomer forms an intramolecular low-barrier hydrogen bond, but to slightly favor the trans isomer in protic solvent. The trans isomer is also favored with the diacid and with the corresponding monoamide, for which normal pK_a-mismatched intramolecular hydrogen bonds are formed by the cis isomers. The cis−trans equilibria were used to estimate the relative strength of the intramolecular hydrogen bonds formed by the cis isomers. The low-barrier hydrogen bond of citraconic monoanion in DMSO is estimated to be about 4.4 kcal/mol stronger than the pK_a-mismatched hydrogen bonds of the diacid and monoamide. These data provide a basis for prediction of the potential differences in strengths of hydrogen bonds between ground state and reactive or transition state complexes in enzyme catalysis.

Recent efforts to explain the stabilization of intermediates or transition states in enzymatic reactions have invoked the importance of low-barrier hydrogen bonds, formed when the pK_a of the hydrogen bond donor matches that of the conjugate acid of the hydrogen bond acceptor.[1−3] Such hydrogen bonds have also been called short strong hydrogen bonds based on the short distance between hydrogen bond donor and acceptor atoms (<2.45 Å when donor and acceptor atoms are oxygen) and the energies of 30 kcal/mol or greater in the gas phase.[1,2,4] Other notable features of low-barrier hydrogen bonds include the low isotopic fractionation factor, the Hadzi type ii IR spectra, and the extreme downfield NMR shift of the proton involved in this hydrogen bond.[4−6] Perrin and co-workers have demonstrated through isotopic perturbation of equilibrium experiments that low-barrier or single-well hydrogen bonds are formed by the monoanions of phthalic and maleic acids in aprotic solvents, while normal double-well hydrogen bonds are formed by these species in aqueous solution.[7,8] Despite evidence for formation of low-barrier hydrogen bonds in solution and in the solid state, quantitative information regarding the strength of these hydrogen bonds is available only from gas-phase measurements and computational models.[4,9] Comparative affinities of acid and amide analogs of acetyl-CoA to citrate synthase have suggested a fairly small difference in strength of a low-barrier hydrogen bond and a pK_a-mismatched hydrogen bond in enzyme−inhibitor complexes.[10] However, no good solution models are available for predicting the expected magnitude of the effect of such changes. In part due to this lack of relevant experimental data, the potential role of low-barrier hydrogen bonds in enzyme catalysis continues to be debated.[11−13]

One of the most well-studied examples of a low-barrier hydrogen bond is that formed by the hydrogen maleate ion **1a**.[14,15] The unusually low pK_a for the first ionization of maleic acid **1c** has been attributed to this very strong hydrogen bond. This paper describes the use of maleic acid and its derivatives as a simple model system for assessment of the relative strengths of normal and low-barrier hydrogen bonds in solution and for probing the effects of changes of solvent medium and hydrogen bond donor and acceptor functionality on hydrogen bond strengths.

Experimental Section

Materials and Methods. Solvents for equilibration experiments were prepared as follows: Water was distilled before use. HPLC grade methanol was used without further purification. Dry dimethyl sulfoxide (DMSO) was prepared according to literature.[16] Chloroform was dried overnight over 3 Å molecular seives before use.

Ratios of cis and trans isomers were analyzed by reverse-phase HPLC, monitored at 245 nm, at which wavelength the cis and trans isomers have approximately equal molar adsorptivities. Relative peak areas were determined using a recording integrator. Five orders of magnitude difference in compound concentrations could be discerned under experimental monitoring conditions. Retention times (in minutes) on a C18 column (5 μm, 4.6 mm i.d. × 25 cm length) under isochratic conditions of 50 mM monobasic potassium phosphate were as follows: maleic acid, 7.7; fumaric acid, 4.1; citraconic acid, 10.4; and mesaconic acid, 5.1. TBA salts gave retention times identical with those of the corresponding diacids.

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Monotetrabutylammonium (TBA) Salts of Citraconic, Mesaconic, Fumaric, and Maleic acids. The TBA salts of the diacids were prepared by combining 1 equiv of the diacid (in water) with 1 equiv of tetrabutylammonium hydroxide (1 M in methanol) from solutions standardized by titration. After neutralization, methanol was removed *in vacuo* and water was removed by lyophilization. ^{1}H-NMR (DMSO-d_6): TBA citraconate δ 0.93, t, 12H, J_{H-H} = 7.4 Hz; 1.30, m, 8H; 1.56, m, 8H; 1.83, s, 3H; 3.17, t, 8H, J_{H-H} = 8.2; 6.06, s, 1H; 21.2, s, 1H; TBA mesaconate δ 0.93, t, 12H, J_{H-H} = 7.3 Hz; 1.29, m, 8H; 1.57, m, 8H; 1.96, s, 3H; 3.17, t, 8H, J_{H-H} = 8.4 Hz; 6.40, s, 1H; TBA fumarate δ 0.93, t, 12H, J_{H-H} = 7.4 Hz; 1.30, m, 8H; 1.57, m, 8H; 3.16, t, 8H, J_{H-H} = 8.4 Hz; 6.36, s, 2H; TBA maleate δ 0.93, t, 12H, J_{H-H} = 7.3 Hz; 1.30, m, 8H; 1.57, m, 8H; 3.16, t, 8H, 3 J_{H-H} = 8.5 Hz; 6.02, s, 2H; 20.0, s, 1H. ^{1}H-NMR ($CDCl_3$): TBA citraconate δ 1.00, t, 12H, J_{H-H} = 7.3 Hz; 1.41, m, 8H; 1.64, m, 8H; 2.02, s, 3H; 3.25, t, 8H, J_{H-H} = 8.6 Hz; 6.29, s, 1H; 20.2, br s, 1H.

Ammonium 1-Carboxy-1-methyl-2-butenamide and Ammonium 2-Carboxy-1-methyl-1- butenamide. Into a dried RB flask containing citraconic anhydride (1 mmol, 0.09 mL) was added ammonia (0.5 M in dioxane, 4.0 mL). The flask was stoppered and allowed to stir for 12 h. Dioxane was removed *in vacuo* leaving a white powder (146 mg, 1.0 mmol). ^{1}H NMR analysis showed the product to be 60% ammonium 1-carboxy-1-methyl-2-butenamide and 40% ammonium 2-carboxy-1-methyl-1-butenamide. ^{1}H-NMR (DMSO-d_6): ammonium 1-carboxy-1-methyl-2-butenamide δ 1.85, s, 3H; 5.86, s, 1H; 7.1, s, 1H; 8.2, s, 1H; ammonium 2-carboxy-1-methyl-1-butenamide δ 1.87, s, 3H; 5.79, s, 1H; 7.3, s, 1H; 8.6, s, 1H.

Isomerization Catalyzed by Thiophenyl Radical. The diacid, monoanion, or monoamide (0.1 or 0.01 mmol) and phenyl disulfide (0.5 mmol, 109 mg) were added to a 2-mL vial equipped with a stir bar. Solvent (1.0 mL) was added and the vial was placed 12 cm from an illuminated 60-W light bulb with stirring. A thermometer was placed next to the vial to monitor temperature (temperature fluctuated from 28 to 30 °C). Samples for analysis (100 μL) were removed, dissolved in water (1 mL), and filtered through glass wool to remove precipitated phenyl disulfide prior to HPLC injection.

Isomerization Catalyzed by Thiourea. The diacid or monoanion (0.1 or 0.01 mmol) and thiourea (1.0 mmol, 76 mg) were placed in a 2-mL vial equipped with a stir bar. Solvent (1.0 mL) was added and the reaction was allowed to stir at room temperature. Analytical fractons were prepared by quenching a 100-μL aliquot of the reaction with benzyl bromide (1.5 M in methanol) for 4 h. Quenching was necessary due to similar retention times for thiourea and fumarate. The aliquot was then dissolved in water and filtered through glass wool to remove insoluble reagents prior to injection.

Results and Discussion

Experiments described here were designed to examine quantitatively the strength of the low-barrier hydrogen bond of maleic acid monoanion **1a** by measuring the equilibrium constant between **1a** and the corresponding trans isomer **1b** (Scheme 1). Comparison with the equilibrium constant between the corresponding cis diacid **1c**, which should form a normal pK_a-mismatched hydrogen bond, and the trans isomer **1d** should provide an assessment of the relative abilities of pK_a-matched low-barrier and normal pK_a-mismatched hydrogen bonds to stabilize the cis isomers **1a** and **1c**. This comparison could also be derived from pK_a data for the cis and trans isomers. However, isomer equilibration permits direct observation of individual cis/trans equilibria and provides a convenient method for studying various effects on the system, including changes in hydrogen bonding functionality.

The monoanions **1a** and **1b** and the corresponding diacids **1c** and **1d** were equilibrated in water and methanol catalyzed by thiourea. The thiourea-catalyzed isomerization did not proceed in aprotic solvents. The citraconic/mesaconic monoanion **2a/2b** and diacid **2c/2d** equilibria were studied in methanol, DMSO, and chloroform, with isomerization catalyzed by the thiophenyl radical generated by photolysis of phenyl disulfide.[17] The methyl substituent of **2a** was not expected to affect the relative pK_a of the two acid groups significantly so that a low-barrier hydrogen bond could still be formed by the cis monoanion **2a**. ^{1}H-NMR of **2a** in DMSO-d_6 and in $CDCl_3$ showed a proton at 20 ppm, supporting the existence of a low-barrier hydrogen bond in this slightly asymmetric system. In the absence of phenyl disulfide no isomerization was observed, demonstrating that direct photoisomerization of the double bond did not occur. Phenyl disulfide-catalyzed isomerization could not be performed in aqueous solution due to the insolubility of phenyl disulfide. Thiourea did not catalyze the isomerization of citraconic and mesaconic acids, even in aqueous solution. Nevertheless, the isomerization of derivatives of **1** in water and methanol and the isomerization of derivatives of **2** in methanol and aprotic solvents provide overlapping sets of data for comparison of the full range of solvent conditions. The ratio of isomers in equilibrated samples was determined by reverse-phase HPLC. HPLC analysis was also used to verify the absence of contaminating isomers in each starting sample. All equilibria were approached from both directions, starting from each pure isomer, to verify that true equilibrium was reached.

Results of equilibration experiments are summarized in Table 1. The diacids **1c/1d** and **2c/2d** favor the trans isomers in all solvents studied. A modest 2.6-fold shift in equilibrium toward cis is observed with **1c/1d** on going from water to methanol and no change is observed with **2c/2d** on going from methanol to DMSO. The monoanions also favor the trans isomers in protic solvent, though slightly less so than the diacids. A small shift of monoanion **1a/1b** toward cis is observed on going from water to methanol, similar to the small change observed with the diacid. On going from methanol to DMSO, a more striking

Scheme 1

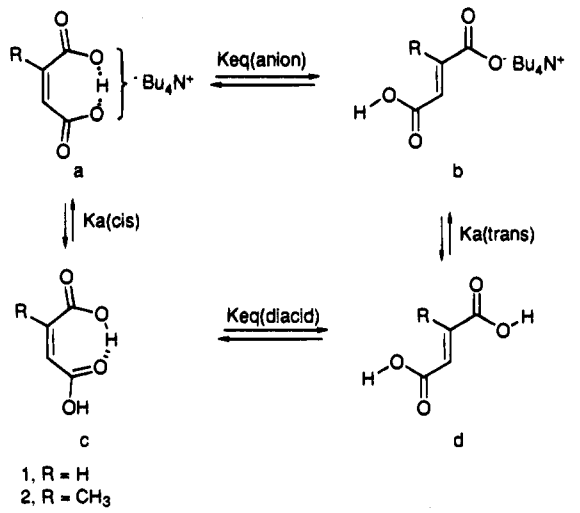

Table 1. Equilibria and Free Energy for Diacid Derivatives[a]

	trans/cis		
compd−solvent	diacid	monoanion	$\Delta\Delta G^b$ kcal/mol
1−H_2O	170:1	73:1	0.5 ± 0.1
1−CH_3OH	65:1	20:1	0.7 ± 0.1
2−CH_3OH	10:1	2:1	1.0 ± 0.1
2−DMSO	10:1	1:160	4.4 ± 0.2
2−$CHCl_3$	ND[c]	1:1050	5.5 ± 0.2[d]
3−DMSO		12:1	4.5 ± 0.2[e]

[a] 0.02 M, 25 °C. [b] The difference in relative trans/cis free energy for the monoanion vs the diacid. [c] Not determined due to insolubility of the diacid. [d] Relative to the diacid equilibrium in DMSO. [e] Relative to the equilibrium for the monoanion **2a/2b** in DMSO.

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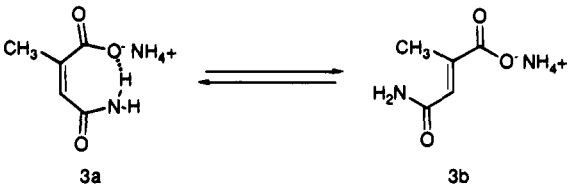
result is observed, as the equilibrium for monoanion **2a/2b** shifts 320-fold toward the cis isomer.[18] A further shift toward cis is observed on going to the less polar aprotic solvent chloroform.[21]

Correlation of observed differences in relative energy of cis and trans isomers to differences in intramolecular hydrogen bond strength requires that intermolecular interactions and ion-pair interactions not be a factor. Literature precedence indicates that ion-pair interactions should not be significant in DMSO at the 0.01 M concentration used, and the use of the tetrabutylammonium cation rather than an alkali metal cation further reduces the likelihood of anion/cation interactions.[22] To verify that ion-pair and intermolecular interactions were not significant, the equilibration experiments were repeated at 0.1 M concentration. The results were identical to those obtained at 0.01 M. This lack of concentration dependence confirms the absence of significant intermolecular interactions. ^{1}H-NMR spectra of **2a** in benzene-d_6 showed shifts in peaks with changes in concentration suggesting intermolecular or ion-pair interactions in this very nonpolar solvent. However ^{1}H-NMR spectra of **2a** in $CDCl_3$ and in DMSO-d_6 showed no such effects at concentrations up to 0.1 M.

The difference in relative free energies of the cis and trans diacids and monoanions ($\Delta\Delta G$ in Table 1) provides an estimate of the relative strengths of the intramolecular hydrogen bonds formed by the cis isomers. Based on the work of Perrin and co-workers, the intramolecular hydrogen bond of **1a** in aqueous solution is expected to have a double potential energy well, due to the nonequivalent solvation of the two carboxylate groups.[7,8] The intramolecular hydrogen bonds of **1a** and **2a** in methanol are presumably also double well for the same reason. The very similar equilibria with **1** in methanol and aqueous solution further support the similarity of intramolecular hydrogen bonding in these two solvents. The large difference in relative cis/trans equilibria for the diacid vs monoanion in DMSO suggests that the low-barrier hydrogen bond of the monoanion is stronger than the pK_a-mismatched hydrogen bond of the diacid by an estimated 4.4. kcal/mol. The very small difference in the same cis/trans equilibria in methanol is consistent with a much smaller difference in relative strengths of pK_a-matched double-well vs pK_a-mismatched hydrogen bonds, though greater stabilization of the negative charge of the carboxylate of the trans isomer in protic solvent may also be a major factor.

The intramolecular hydrogen bond of **2a** appears to increase in strength by an additional 1.1 kcal/mol on going from DMSO to the less polar solvent chloroform. It is difficult to rule out that the 0.1% trans isomer **2b** observed in chloroform might be due to a small amount of contaminating diacid or dianion, either of which would exist as primarily the trans isomer, or by small amounts of each present in equilibrium with the monoanion. The estimate of 5.5 kcal/mol for the low-barrier vs normal hydrogen bond in chloroform is viewed as a lower limit. It is likely that even larger hydrogen bond energies may be achieved in lower dielectric constant medium. Efforts to extend the measurements to solvents of lower polarity were hampered by intermolecular interactions even at low concentration, as evidenced by the concentration-dependent NMR spectra of

Scheme 2



citraconic and mesaconic acid monotetrabutylammonium salts in nonpolar solvents.

As an additional comparison, the monoanions of the corresponding amides **3a** and **3b** were studied in DMSO (Scheme 2). The trans isomer was favored, with an equilibrium constant near that of the diacids **2c** and **2d**. The relative equilibrium cis/trans ratios for **2a/2b** vs **3a/3b** perhaps provide a better indication of the extent to which the carboxylate anion is stabilized by a low-barrier hydrogen bond (in **2a**) vs a normal hydrogen bond (in **3a**) in aprotic solvent. The ratios of cis/trans isomers correspond to a 4.5 kcal/mol difference in relative cis/trans free energy ($\Delta\Delta G$). This is consistent with a greater strength of the low-barrier hydrogen bond of **2a** relative to the normal hydrogen bond of **3a**. Such comparison of acid vs amide functionality could not be made from pK_a data and demonstrates an advantage of isomer equilibrium measurements. These data may provide a basis for predicting the effects on enzyme activity and/or substrate binding upon acid to amide mutagenesis of residues involved in key hydrogen bonding interactions.

Assignment of the observed differences in relative energy of cis and trans isomers to differences in intramolecular hydrogen bond strength are complicated by uncertainties in effects of solvent interactions. However, changing the low-barrier hydrogen bond of **2a** to a normal one by decreasing the basicity of the hydrogen bond acceptor (in the cis diacid **2c**) and by decreasing the acidity of the hydrogen bond donor (in **3a**) gives similar results. These combined data provide strong evidence for the 4.4 kcal/mol greater strength of the low-barrier hydrogen bond of **2a** in DMSO relative to a normal pK_a-mismatched hydrogen bond.

The strengths of hydrogen bonds are known to increase with increasing acidity of the donor and increasing basicity of the acceptor, with the strongest hydrogen bonds formed when the pK_a of the donor matches that of the conjugate acid of the acceptor.[23-25] The above results provide an estimate of the magnitude of the increase. An additional issue of current interest is whether there is some special stability of low-barrier hydrogen bonds, in addition to the increased strength expected based on pK_a matching. The 3.4-kcal/mol difference in relative cis/trans free energy between **2a/b** in methanol vs DMSO could be explained by a greater strength of the low-barrier hydrogen bond in DMSO relative to the pK_a-matched but double-well hydrogen bond in protic solvent. Alternatively, this result could be explained by better solvation of the ionized carboxylate of the trans monoanion in methanol than in DMSO. Theoretical calculations have been used to argue against any special stability of hydrogen bonds associated with a disapperance of the barrier to proton transfer.[12] It appears that favorable covalent forces and electrostatic attraction of low-barrier hydrogen bonds are cancelled by unfavorable steric repulsion.[9] The theoretical analysis is based on systems in which the hydrogen bond length

(18) Relative cis/trans equilibria deduced from published pK_a values of 3.9 and 7.3 for maleic and fumaric acids, respectively, in pyrollidinone[19] are in good agreement with the relative cis/trans ratios measured in DMSO, though differing pK_a values have been reported.[20]

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is not enforced by external forces. The results reported here may be influenced by the fact that the intramolecular hydrogen bonds of maleic and citraconic acid derivatives are forced to be short by the rigid geometry of the molecule. This may have a negative effect on the strengths of hydrogen bonds under pK_a mismatching or protic solvent conditions by enforcement of steric repulsion in the absence of optimal covalent forces. However, enzymes might use a similar tactic, forcing hydrogen bonding groups together by distant binding interactions in order to enhance the strength of a pK_a-matched low-barrier hydrogen bond in an intermediate or transition state complex relative to a pK_a-mismatched hydrogen bond in the enzyme−substrate complex.

The difference in hydrogen bond strengths between a pK_a-matched low-barrier hydrogen bond vs pK_a-mismatched hydrogen bonds appears to be only about 4.4 kcal/mol, regardless of whether the low-barrier hydrogen bond is stronger than a pK_a-matched but double-well hydrogen bond. The intramolecular hydrogen bond in **2a** may not be the strongest possible low-barrier hydrogen bond, perhaps due to non-optimal steric constraints and/or the fact that this hydrogen bond is formed between the less basic anti-orbitals of the carboxylates. Determination if stronger low-barrier hydrogen bonds can be formed will require further study.

The method developed in this study provides a basis for assessment of the relative energies of normal and low-barrier hydrogen bonds in polar aprotic media. This information should be complementary to gas-phase data in predicting the energy of stabilization of enzyme-bound intermediates or transition states that may be provided by formation of low-barrier hydrogen bonds. Such information has been lacking in previous arguments regarding implications of low-barrier hydrogen bonds in enzyme catalysis. These results, along with approximations recently made with citrate synthase inhibitor complexes, provide evidence that low-barrier hydrogen bonds are stronger than pK_a-mismatched asymmetric hydrogen bonds, but that the relative energy differences in enzyme active sites and in solution are much smaller than observed in the gas phase.[10] It is also possible that the energy may be somewhat higher than these estimates in the low dielectric constant environment of an enzyme active site, or even in different model systems. However, these results should begin to provide estimates of potential strengths of low-barrier hydrogen bonds in solution, as a valuable complement to gas-phase data.

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