→ P) increase with the strength of binding of ROH to β-CD, as was found with pNPA.[4] Using an approach discussed elsewhere,[5b,11] we can estimate apparent dissociation constants (K_{ts}) for the transition states containing ROH.[12] Values of K_{ts} also parallel K_i (Table I), and there is a reasonable correlation between pK_{ts} and pK_i (Figure 2b), even though the alcohols include different structural types. These trends are consistent with modes of binding of ROH in the ternary complexes *and* in the transition states for cleavage that are not too dissimilar from those in the β-CD·ROH complexes.

Values of k_t are 1.4–4.3 times larger than $k_c = 0.14\ s^{-1}$ for CD·pNPH[8a] since the catalysis by ROH is only modest. Nevertheless, they must mean that the presence of an alcohol in the β-CD cavity can stabilize the transition state relative to the initial state.[11] Presumably, the alcohols act as inert spacers,[13] improving the fit of the acyl chain of pNPH in the cavity of β-CD in the transition state for ester cleavage.[14] This behavior may be considered a novel type of "spectator catalysis".[15] It will be of interest to see how this form of catalysis varies with the substrate, the CD, and the structure of PI.[16]

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Structural Model of a Short Carboxyl–Imidazole Hydrogen Bond with a Nearly Centrally Located Proton: Implications for the Asp-His Dyad in Serine Proteases

Richard D. Gandour,* Nabeel A. R. Nabulsi, and Frank R. Fronczek

Department of Chemistry, Louisiana State Univerity Baton Rouge, Louisiana 70803-1804

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The cornerstone of the hypothesis[1] on the orientation of carboxylate in general base catalysis is that there is more electron density in the syn direction than in either anti.[2] Consequently, when carboxylate hydrogen bonds to an acid with a pK_a comparable to that of carboxyl, the position of the proton should depend on the directionality of the hydrogen bond to carboxylate.[3] If anti, the proton will be closer to the weak base; if syn, closer to the carboxylate. As ΔpK_a between donor and acceptor approaches 0, the hydrogen bond becomes equidistant and the distance between the heavy atoms decreases.[4]

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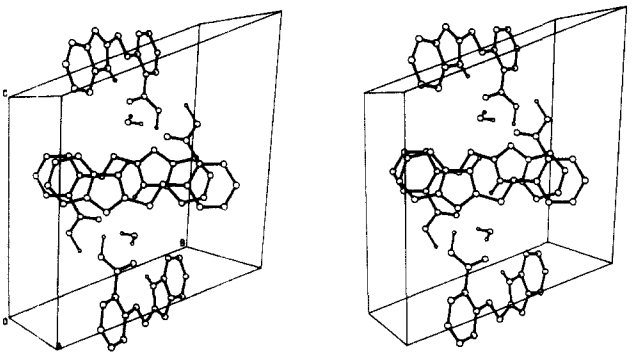


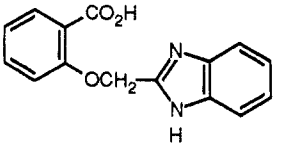
Figure 1. Stereoview of the packing diagram of 2-(2-benzimidazolylmethoxy)benzoic acid.

Table I. Hydrogen-Bonding Parameters[a]

atoms	O···N, Å	O–H, Å	O–H···N, deg
O2A–N1B	2.590 (2)	1.18 (3)	175 (3)
O2B–N1A	2.594 (2)	1.16 (2)	177 (2)
mean	2.592 (2)	1.17 (2)	176 (2)

[a] Standard deviations are in parentheses.

Single-crystal X-ray analysis of small-molecule models of biomolecular hydrogen bonds reveals structural trends[5,6] that parallel the trends in proteins.[7,8] The 100-fold improvement in resolution in the smaller structures enables a more precise picture of these trends.



1

In the crystal structure of $\mathbf{1}{\cdot}1/2H_2O$ (see Figure 1), two crystallographically independent molecules form intermolecular hydrogen bonds between carboxyl and benzimidazolyl as chains along the direction of the *c* axis.[9] The two hydrogen bonds are independent measures of the same interaction (Table I). The resulting O···N contacts are shorter than the mean O···N distances of imidazolium–carboxylate couples.[10] A water bridges the two molecules by hydrogen bonding to the carboxyls, with O···O distances 2.756 (3) and 2.793 (3) Å.

The strong *inter*molecular hydrogen bonding between a syn-oriented carboxyl and a benzimidazolyl, rather than anti-oriented *intra*molecular hydrogen bonding, emphasizes the importance of orientation. Hydrogen bonds between carboxyl and imidazole in small molecules[11] and in proteins[8] strongly prefer the syn orien-

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(10) (a) Gorbitz[6] reports two means: 2.664 (17) Å for (His)HN_π^+···$^-$OOC–(Asp/Glu) and 2.736 (35) Å for (His)HN_τ^+···$^-$OOC–(Asp/Glu). The crystal structure of histidinium trimesate[10b] has a shorter (His)HN_π^+···$^-$OOC– bond (2.568 (7) Å; $R = 0.057$), but the hydrogen positions were not accurately determined. (b) Herbstein, F. H.; Kapon, M. *Acta Crystallogr., Sect. B* **1979**, *B35*, 1614–1619.

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tation. In this crystal structure, the preference for syn versus anti dominates over any preference for *intra*molecular versus *inter*molecular.

The central location of the proton in the hydrogen bond suggests nearly equivalent pK_a's. Syn-anti directionality and microenvironmental electrostatics control carboxylate basicity. In water, benzimidazole is a stronger base than carboxylate.[12-16] The pK_a of carboxyl increases in less aqueous solvents and exceeds that of imidazolium.[17,18] In anhydrous environments, the Asp-His dyad will favor the neutral form rather than the zwitterionic. This point is illustrated by the crystal structure of a carboxylic acid-imidazole clathrate, which favors the zwitterion when hydrated and the neutral form when anhydrous.[19] The zwitterionic dyad has an anti-oriented carboxylate, but the neutral form has a syn-oriented carboxyl.

These results inform the controversy about charge-relay catalysis in serine proteases. Structural studies on these enzymes[20-22] suggest a zwitterionic dyad with a syn-oriented carboxylate. Most believe that the proton does not transfer from imidazolium to carboxylate and the dyad remains zwitterionic. Warshel et al.[23] propose that an ionized Asp electrostatically stabilizes the transition state. The pK_a of His increases during the enzymatic reaction,[24] and a proximal carboxylate increases the basicity of imidazole.[25]

Proton-inventory studies suggest two-proton catalysis for hydrolysis of natural substrates.[26-28] Schowen[29] questions the use of equilibrium data[20-22] as evidence for a zwitterion in the transition state. Substrate binding may change the protein microenvironment to favor repositioning or transfer of the proton in the dyad.[28]

The crystal structure of a nearly centrally located proton in a carboxyl-imidazole hydrogen bond suggests that a similar structure can exist in a transition state. Repositioning of the proton in a broad single-potential well[30] would produce a curved proton inventory just as proton transfer would.[31] The dyad remains zwitterionic, but at the transition state the O···N distance shortens and the proton moves to center. Proton transfer is not required for catalysis; strong hydrogen bonding will suffice.[32] This catalytic hydrogen-bonding mechanism reconciles the kinetic, structural, and computational results presented to date.

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Supplementary Material Available: Tables of atomic coordinates for **1**·$^1/_2H_2O$, bond distances, and bond angles and ORTEP drawings of molecules A and B (8 pages). Ordering information is given on any current masthead page.

(12) The individual K_a's of **1** in 80% methanol-water are too close to separate by titration.[13] The first ionization is mostly imidazolium, and the second is mostly carboxyl. The pK_a of 2-methoxybenzoic acid[15] is 4.09 in water. The pK_a of 2-(phenoxymethyl)benzimidazole is 4.34 in 48% ethanol-water.[14] From the pK_a's of other benzimidazoles in 48% ethanol-water versus water, we estimate a pK_a of 4.68 for **1** in water.

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Copper Coordination Geometry in Azurin Undergoes Minimal Change on Reduction of Copper(II) to Copper(I)

William E. B. Shepard, Bryan F. Anderson, David A. Lewandoski, Gillian E. Norris, and Edward N. Baker*

Department of Chemistry and Biochemistry Massey University, Palmerston North, New Zealand

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It has long been suggested[1] that the copper coordination environment in blue copper electron transfer proteins[2] is a compromise between preferred states for copper(I) and copper(II), and that this should lower the activation energy for electron transfer. Here we provide direct crystallographic evidence that the copper site in azurin, from *Alcaligenes denitrificans*, undergoes minimal structural change on reduction, consistent with the requirements for fast electron transfer. This conclusion is based on crystallographic refinement of the reduced, copper(I), azurin structure, at 1.9-Å resolution,[3] and comparison with the structure of the oxidized protein, at similar resolution.[4,5]

Azurin was isolated and purified from cultures of *A. denitrificans* (NCTC 8582) and crystallized in its oxidized, Cu(II), state as described previously.[6] These crystals were then reduced by soaking in standard mother liquor (0.1 M phosphate, 75% saturated with ammonium sulfate) containing 0.1 M ascorbic acid, the final pH of this solution being 5.5. Reduction appeared complete in 4-5 h, but soaking was extended until 7 h. This procedure resulted in small changes in the unit cell dimensions

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