Proc. Natl. Acad. Sci. USA
Vol. 77, No. 6, pp. 3095–3097, June 1980
Chemistry

Benzoate catalysis in the hydrolysis of *endo*-5-[4′(5′)imidazolyl]-bicyclo[2.2.1]hept-*endo*-2-yl *trans*-cinnamate: Implications for the charge-transfer mechanism of catalysis by serine proteases

(¹³C and ¹⁵N NMR spectroscopy/ester hydrolysis/base catalysis/medium effects on reaction rates/enzyme mechanisms)

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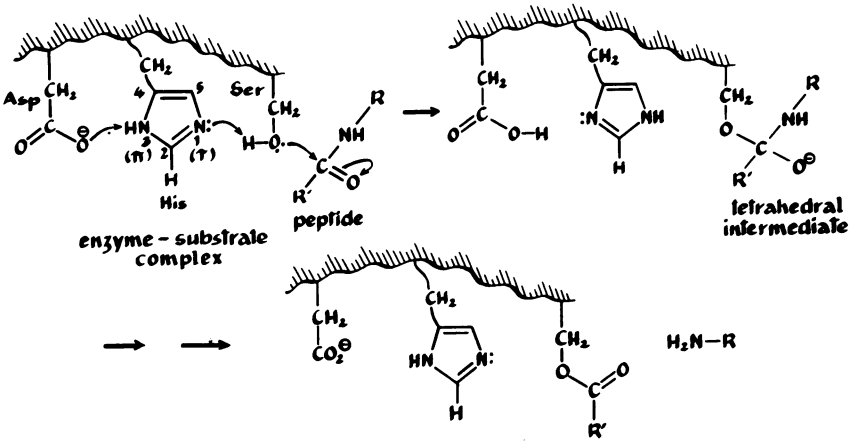
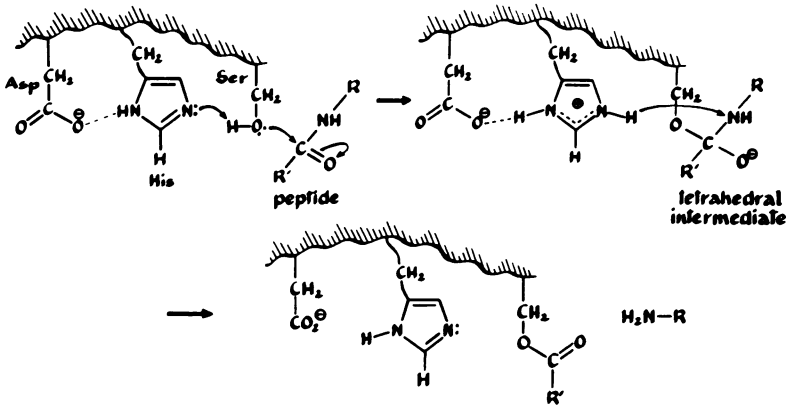
Contributed by John D. Roberts, February 19, 1980

ABSTRACT **The acceleration, by a factor of 2500, of the hydrolysis of *endo*-5-[4′(5′)imidazolyl]bicyclo[2.2.1]hept-*endo*-2-yl *trans*-cinnamate by 0.5 M sodium benzoate in 42 mol % dioxane in water can be explained without resort to operation of a "charge-relay" mechanism similar to that often postulated to account for the enzymatic activity of serine proteases. The degree of ionization of 4-methylimidazole and of sodium benzoate in 42 mol % dioxane in water at 60°C have been measured by NMR spectroscopy.**

A "charge-relay" mechanism has been postulated to be crucial to the effectiveness of operation of the "catalytic triad" (serine, histidine, and aspartic acid residues) in the enzymatic cleavage of peptide bonds by serine proteases (1). The key feature of this mechanism (Eq. 1) is formation of a tetrahedral intermediate by attack of serine on the peptide carbonyl, assisted by proton transfers from serinyl OH to histidyl imidazole and of H3 of the histidyl imidazole to the aspartate carboxylate anion. That the effectiveness of the second of these transfers requires the aspartate carboxylate to be a stronger base than the histidyl imidazole has been clearly delineated by Hunkapiller *et al.* (2). However, a ^{15}N NMR investigation (3) of α-lytic protease clearly showed that the histidyl imidazole of this enzyme has a normal base strength and the decrease of activity that occurs at low pH values is fully consistent with protonation of this imidazole rather than the aspartate carboxylate anion of the triad (2). An alternative mechanism (Eq. 2) has been proposed (3), which might be called a carboxylate-assisted process. This mechanism features (*i*) a sufficient degree of hydrogen bonding of histidyl imidazole in the tautomeric form with the proton on N3 and the aspartate carboxylate to make this tautomer the predominant one, and (*ii*) assistance of transfer of the serine proton to N1 of the imidazole by both hydrogen and electrostatic bonding between the resulting imidazolium cation and aspartate carboxylate. This mechanism is completed to the serine ester stage by transfer of a proton from the imidazolium cation to the nitrogen of the leaving RNH— group (3).



[1]



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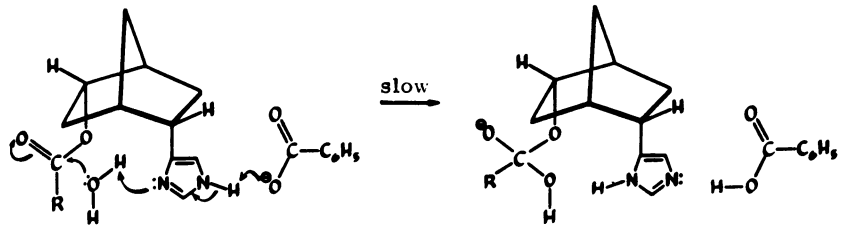

[3]

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The elegant model compound studies by Bender and coworkers (4, 5), however, seem to provide strong support for the charge-relay formulation through discovery of a very pronounced benzoate catalysis of the hydrolysis of *endo*-5-[4′-(5′)imidazolyl]bicyclo[2.2.1]hept-*endo*-2-yl *trans*-cinnamate (I). The postulated mechanism is shown in Eq. 3, with water playing the role of serine, the pendant imidazole the role of histidine, and benzoate ion the role of the aspartate carboxylate. The argument for this mechanism is derived from the fact that, although there is no benzoate catalysis, even at 0.5 M benzoate in water, there is a steadily increasing catalytic effect with increasing dioxane content in the water. This increase in catalytic effectiveness of benzoate ion can be attributed to an increase in the pK_a of the benzoic acid and, thus, a concomitant increase in the basicity of the benzoate ion (5). Exactly the same kind of argument has been used to account for the postulated effectiveness of the aspartate carboxylate in the charge-relay mechanism—the carboxylate in question being located in a "hydrophobic pocket" of the enzyme (1), thus leading to the expectation of a larger pK_a and greater basicity of the aspartate carboxylate (2).

We believe that there are alternative interpretations of the results. Benzoate ion in 42 mol % dioxane in water has a high catalytic effectiveness. The ratio of catalyzed rate to uncatalyzed rate is about 400 for 0.1 M benzoate (2500 for 0.5 M benzoate) between pH 6 and 8, where the reported pK_a is 9.4 (5). Nonetheless, the reported pK_a value of 9.4 implies that the effective concentration of benzoate ion must be small (calculated for a 0.1 M solution to be 0.004 M at pH 8 and 4×10^{-5} M at pH 6). We have used ^{13}C and ^{15}N NMR to probe the species present in the solutions at these concentrations with 4-methylimidazole as model for I.

Table 1. 4-Methylimidazole and benzoic acid (0.5 M each) in 42 mol % dioxane/58% H_2O at 60°C

		Benzoate		4-Methylimidazole		
				δ ^{15}N, ppm*		% imidazolium
pH	μ	δ ^{13}C, ppm‡	% $C_6H_5CO_2^-$§	N1	N3	cation†
6.1	0.5¶	−141.0	56.6	190.6	183.5	60.1
6.4	0.5¶	−141.8	71.7	188.6	180.9	52.9
6.7	0.5¶	−142.2	79.2	185.9	177.9	44.0
6.1	0.1‖	−140.2	41.5	189.4	182.5	56.6

* Upfield from 1 M $H^{15}NO_3$.

† Calculated from the following ^{15}N shifts of 0.5 M 4-methylimidazole (μ = 0.1) in 42 mol % dioxane/58% H_2O:

		δ ^{15}N, ppm	
	pH	N1	N3
4-methylimidazolium cation	4.3	202.0	197.7
4-methylimidazole	8.5	174.2	161.1

‡ Shift of carboxyl carbon downfield of the methyl carbon of *tert*-butyl alcohol.

§ Calculated from the ^{13}C shifts of carboxyl carbon of 0.1 M benzoic acid in Fig. 1.

¶ $C_6H_5COO^-Na^+$ (0.5 M) and 4-methylimidazole (0.5 M) dissolved in dioxane/H_2O and adjusted to pH by addition of HCl.

‖ C_6H_5COOH (0.5 M), 4-methylimidazole (0.5 M), and KCl (0.1 M) dissolved in dioxane/H_2O and adjusted to pH by addition of NaOH.

EXPERIMENTAL SECTION

The ^{13}C NMR spectra were determined at 25.14 MHz with a Varian XL100 spectrometer using the pulse Fourier transform technique with a 35-μsec pulse width and 3-sec repetition rate with proton decoupling, and 12-mm sample tubes. *tert*-Butyl alcohol and ^2H_2O in a capillary provided both the shift reference and the lock signal. The ^{15}N NMR spectra were obtained with a Bruker WH 180 spectrometer operating at 18.25 MHz, a 55-μsec pulse width, 5-sec delay, and full proton decoupling (6). To remove traces of Fe^{3+} and Cu^{2+}, the dioxane was distilled from 8-hydroxyquinoline. This procedure ensured being able to detect the nitrogen resonances of 4-methylimidazole (3, 7).

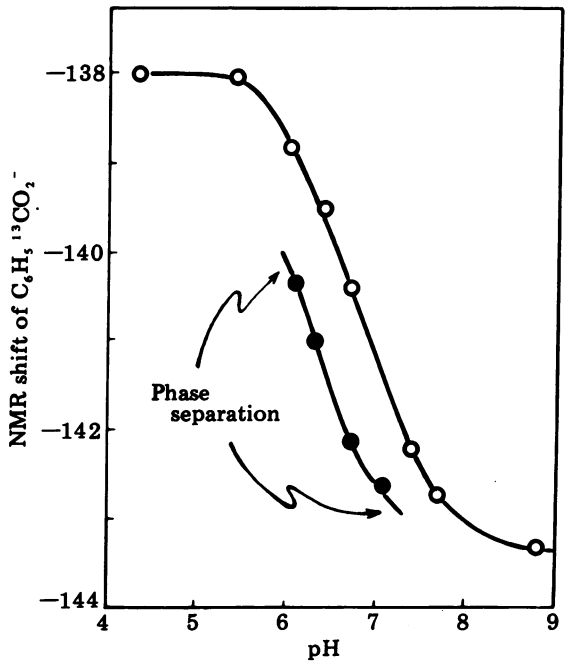
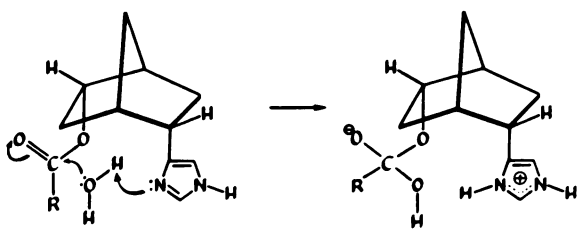


FIG. 1. ^{13}C NMR chemical shifts of benzoate carboxyl carbon as a function of pH at 60°C in 42 mol % dioxane in water. ○, 0.1 M C_6H_5COOH with 0.1 M KCl; the pH was changed by addition of NaOH. ●, 0.5 M $C_6H_5COO^-$ Na^+; the pH was adjusted by addition of HCl. The pH values for this solvent mixture are corrected from glass electrode readings by the procedure of Van Uitert and Fernelius (9). The shifts are in ppm downfield of the methyl carbons of *tert*-butyl alcohol. The curves were calculated for pK_a values of 6.78 and 6.2 for 0.1 M and 0.5 M benzoate concentrations, respectively.

RESULTS AND DISCUSSION

The use of ^{13}C and ^{15}N NMR spectroscopy to determine the degree of ionization of carboxylic acids and imidazoles, respectively, is well established (3, 7, 8). The results obtained for several solutions in 42 mol % dioxane in water containing benzoic acid and 4-methylimidazole at different pH (9) and ionic-strength values are given in Table 1. Clearly, the concentration of benzoate ion in these solutions greatly exceeds that which might be expected from the reported pK_a value. The discrepancy is more apparent than real because the reported pK_a is the thermodynamic value derived with proper regard for the activity coefficients of the ions in this solvent system (5, 10). Concentration equilibrium constants for benzoic acid corresponding to pK_a values of 6.78 and about 6.2 can be derived, respectively, for 0.1 M solutions (with an approximate ionic strength of 0.1) and 0.5 M solutions (ionic strength, 0.5) from the ^{13}C NMR titration curves of Fig. 1. The pK_a values were not changed significantly by addition of equivalent concentrations of 4-methylimidazole. The curve in Fig. 1 for ionic strength 0.5 could not be determined over the whole range of concentrations because of phase separations, but, unquestionably, there is the expected substantial decrease in pK_a with increasing ionic strengths. The pK_a of 4-methylimidazolium cation calculated from the concentrations listed in Table 1 is 6.4 ± 0.2, which is, within experimental error, the same as the thermodynamic value for I reported by Komiyama *et al.* (5). This is as expected for the ionization of a cationic acid, $BH^+ + H_2O \rightleftarrows B + H^+_3O$, where changes in the activity coefficient of BH^+ and H^+_3O with solvent and ionic strength will be reasonably parallel.

The hydrolysis rate of I substantially decreases in the absence of benzoate as the proportion of dioxane is increased in the solvent. This decrease has been attributed to preferential solvation of the *trans*-cinnamate group by dioxane, causing steric hindrance to the concerted attack of water and imidazole, as in Eq. 3. A more important factor is likely to be the decreased ability of the increasingly nonpolar solvent to accommodate the charge separation produced in the formation of the transition state for Eq. 4. In order for benzoate ion to be effective



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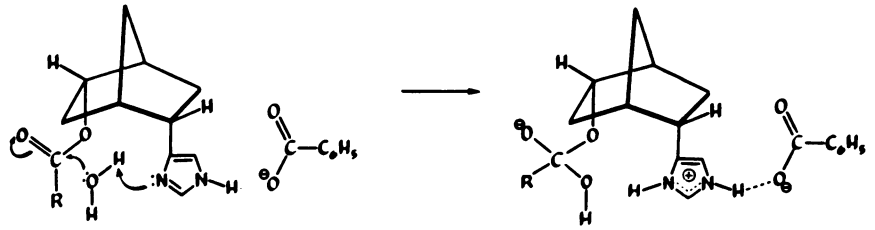
in the mechanisms such as Eq. 3, it should be of comparable basicity to the imidazole group, and, yet, even in 17% mol fraction dioxane in water, where benzoate is only 1/10th as strong a base as imidazole, the rate of 0.5 M benzoate is about 300 times faster than when benzoate is absent (5).

It is our view that benzoate ion can increase the hydrolysis rate of I by virtue of acting much like the asparate carboxylate group in the catalytic triad of a serine protease: first, by hydrogen bonding to the imidazole before the transition state, which will make the imidazole ring more nucleophilic, and, second, by additional stabilization of the transition state by a further degree of hydrogen bonding associated with building up the imidazolium charge as the result of proton transfer (Eq. 5). In addition, there are likely to be secondary influences of benzoate ion at these large concentrations which contribute to the general polarity of the medium. That the combination of adding benzoate and dioxane could make the reaction rate faster than in pure water is in accord with the speculations of Warshel (11). Formulations such as given in Eq. 5 also are supported by the failure to obtain evidence for a two-proton



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transfer mechanism in the benzoate-assisted hydrolysis of I,* although such two-proton transfers have been suggested by proton-inventory studies for some, but not all, reactions catalyzed by α-lytic protease (12) and trypsin (13).

We thank Profs. J. H. Richards and W. W. Bachovchin for many helpful suggestions. This is contribution no. 6173 from the Gates and Crellin Laboratories of Chemistry. These studies were supported by U.S. Public Health Service Grant GM-11072 from the Division of General Medical Sciences and by the National Science Foundation.

* Bender, M. L., presented at the Fourth IUPAC Conference on Physical Organic Chemistry, York, England, September 4–8, 1978.

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