Solvent Isotope Effects on a Hydrolysis Reaction Catalysed by Subtilisin and its N155G Mutant. Failure of the Proton Inventory Method to report Hydrogen-bonding Interactions in the Oxyanion Hole

Y. Chiang,[a] A. J. Kresge,*[a] T. K. Chang,[b] M. F. Powell[b] and J. A. Wells[b]

[a] *Department of Chemistry, University of Toronto, Toronto, Ontario M5S 1A1, Canada*
[b] *Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080, USA*

Proton inventories (solvent isotope effects in H_2O–D_2O mixtures) are not detectably different for the hydrolysis of an anilide substrate catalysed by subtilisin and its N155G mutant, despite the fact that this mutation removes a critical hydrogen-bonding interaction supplied by Asn-155 in the oxyanion hole of the enzyme, which stabilizes the transition state of this reaction by an amount equivalent to two orders of magnitude in reaction rate.

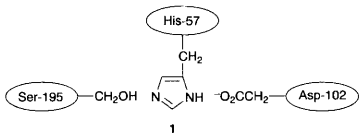
The interpretation of solvent isotope effects in H_2O–D_2O mixtures, commonly called 'proton inventories', has led to an intriguing hypothesis concerning the role of the catalytic triad **1** and the operation of the charge-relay mechanism in the serine protease-catalysed hydrolysis of ester and amide substrates. It has been suggested that, whereas the reaction of simple substrates involves only a single proton transfer, that from the hydroxy group of Ser-195 to the basic nitrogen atom of the imidazole side chain of His-57, the hydrolysis of large substrates whose structures complement the specificity pockets of the enzymes involves a second proton transfer from the other nitrogen atom of the imidazole of His-57 to the carboxylate group of Asp-102 (chymotrypsin numbering).[1] Specific substrates thus activate the presumably more efficient double-proton transfer, charge-relay mechanism. This idea has interesting implications for the evolutionary development of enzyme catalytic efficiency and substrate specificity.

A critical study, however, has cast doubt upon the validity of this hypothesis.[2] It was found that proton inventories of reactions catalysed by chymotrypsin whose His-57 imidazole hydrogen had been replaced by a methyl group, and the second proton transfer and charge-relay catalysis were thus clearly precluded, still indicated that more than one proton was playing an active role in the process. The hypothesis was advanced that these proton inventories were signalling the formation of hydrogen bonds between the substrate and groups situated in the oxyanion hole of the enzyme.[3] We wish to report that we have examined a reaction catalysed by an enzyme in which the hydrogen-bonding ability of the oxyanion hole has been impaired by site-directed mutagenesis and we have found no detectable difference between the proton inventory for this reaction and that catalysed by the wild-type enzyme with an intact oxyanion hole. Hydrogen-bonding interactions in the oxyanion hole, although they do contribute significantly to the catalytic efficiency of the enzyme, thus appear not to be reflected in proton inventories.

The proton inventory method is based upon a formalism that relates solvent isotope effects, k_x/k_H, to the atom fraction of deuterium in the solvent, x in terms of fractionation factors, ϕ, according to the general expression shown in eqn. (1).[4]†

$$\frac{k_x}{k_H} = \frac{\Pi_i (1 - x + x\phi_i^{\ddagger})}{\Pi_j (1 - x + x\phi_j^{is})} \quad (1)$$

Fractionation factors are partial isotope effects expressed as D : H ratios at particular sites relative to the D : H ratio of the solvent; the terms in eqn. (1) include fractionation factors for all exchangeable sites in the transition state, $\phi_i^{\ddagger}$, and all exchangeable sites in the initial state, ϕ_j^{is}. Fractionation factors reflect the tightness of the bonding of the hydrogens they represent; they are significantly different from unity for hydrogens being transferred, where they represent primary isotope effects, and also for hydrogens in strong hydrogen bonds, whose overall bonding is loose.[5] Mechanistic inferences are drawn from the shape of the relationship between k_x/k_H and x, from which changes in fractionation factors between the initial state and the transition state might be deduced and consequently the number of hydrogens being transferred or engaged in strong hydrogen bonding might be determined.



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Deviations from a linear relationship between k_x/k_H and x are seldom very strong, and experimental results can usually tell only if the dependence is linear or curved, but not the nature of the curvature. Since the deviation from linearity is greatest at $x = 0.5$, we have, following Albery,[6] performed our proton inventory measurements in this region, dubbed 'HDO', as well as at the limits $x = 0$ (H_2O) and $x = 1$ (D_2O).

The hydrogen-bonding interactions in the oxyanion hole of chymotrypsin are provided by N–H bonds of two main-chain amide groups, and these interactions cannot be eliminated by removing these groups without severely changing the structure of the enzme. In subtilisin, on the other hand, one of the interactions involves the side-chain amide group of Asn-155, and this can be removed by replacing Asn-155 with other amino acids that have no side-chain groups capable of hydrogen bonding, and still leave a functioning enzyme, albeit one with greatly reduced activity,[7,8] We consequently performed our study by comparing proton inventories for subtilisin and its N155G mutant in which Asn-155 is replaced by Gly.

We measured the rates of hydrolysis of the peptide substrate succinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide by monitoring the appearance of *p*-nitroaniline spectroscopically. First-order V_m/K_M rate constants were determined by making measurements at low substrate concentrations, $[S]_0$ = (0.004–0.02) K_M, and zero-order initial rates were also determined by making measurements with substrate concentrations in the vicinity of K_M, $[S]_0$ = (0.05–15) K_M; the latter were then fitted by non-linear least-squares analyses to the Northrop form[9] of the Michaelis–Menten equation to provide values of V_m and V_m/K_M. It was found that V_m/K_M rate constants obtained from Michaelis–Menten fitting showed more scatter than those determined under first-order conditions, and only the latter were therefore used in constructing proton inventories. Measurements in H_2O, HDO and D_2O were made in sets using the same enzyme stock solution for all three solvents in order to ensure equal enzyme concentrations within a given set. Each set for Michaelis–Menten fitting consisted of *ca.* 30 zero-order rate constants for each of the three solvents, and each set of first-order rate constants consisted of 3–8 rate constants for each solvent. The V_m proton inventories are based upon 10 sets of measurements for the wild-type enzyme and 14 sets for the mutant, and the V_m/K_M proton inventories are based upon 9 wild-type sets and 3 mutant sets. Measurements were made in Tris buffers at pH = 8.2, 8.5, 8.6 and 8.9, and in carbonate buffers at pH = 10.0, and enzyme stock solutions were prepared with either H_2O, HDO or D_2O or D_2O as the solvent; none of these differences in reaction conditions affected the

proton inventories. The data are displayed in Fig. 1 and are available in supplementary tables from the authors upon request.

Each of these proton inventories is fitted well by a quadratic expression: least-squares analysis gives the following relationships: $(V_m/K_M)_x/(V_m/K_M)_H = 1 - (0.054 \pm 0.019)x - (0.236 \pm 0.021)x^2$ for the wild-type enzyme and $1 - (0.019 \pm 0.042)x - (0.297 \pm 0.047)x^2$ for N155G, and $(V_m)_x/(V_m)_H = 1 - (0.763 \pm 0.003)x + (0.093 \pm 0.037)x^2$ for wild-type and $1 - (0.757 \pm 0.022)x + (0.115 \pm 0.024)x^2$ for N155G. There are no significant differences between the coefficients of these expressions for the wild-type and mutant enzymes for either the V_m/K_M or V_m relationships, and both kinds of proton inventory must therefore be considered to be the same for both enzymes. This is so despite the fact that the difference in hydrogen-bonding interaction between the two enzymes is highly significant: removing it in the mutant reduces both V_m/K_M and V_m by two orders of magnitude.[7c,d]‡

Mechanistic interpretation of these proton inventories is restricted by the fact that, whereas the V_m/K_M and V_m rate constants refer to different initial states, free enzyme and substrate in the former case and enzyme–substrate complex in the latter, both refer to the same transition state. This follows from general considerations of enzyme kinetics, which show that V_m/K_M consists of rate constants for all steps up to and including the first irreversible step whereas V_m consists of rate constants for all steps following enzyme–substrate binding up to and including the rate-determining step,[10] and the fact that acylation of the enzyme is both rate-determining[11] and irreversible in subtilisin-catalysed hydrolysis of amide substrates. The transition-state term of eqn. (1) must therefore be the same for both the V_m/K_M and V_m proton inventories.

Even with this restriction, a number of different interpretations of the data are possible. For example, the V_m results fit a model with two different transition state fractionation factors, $\phi_1^{\ddagger} = 0.42 \pm 0.03$ and $\phi_2^{\ddagger} = 0.82 \pm 0.05$, consistent with the conventional double proton-transfer mechanism.[1] This picture, however, must be elaborated because of the dome-shape and weaker overall isotope effect of the V_m/K_M inventory. This can be done in a variety of ways, for example, by introducing the initial state (free enzyme plus substrate) medium effect, $\Phi^{is} = 0.49 \pm 0.01$. This represents the aggregate influence of a large number of sites in the enzyme and substrate and/or their solvation shells that undergo small changes in fractionation factor between initial state and transition state, *e.g.* 40 sites with an average change of $\Delta\phi = 0.02$. Such a medium effect could be provided, for example, by a conformational change as the enzyme and substrate form the enzyme–substrate complex. This interpretation, however, is not unique for good fits of the data may be obtained with one, three, four, or more transition state fractionation factors modified by initial-state medium effects for both the V_m and V_m/K_M inventories. Other, different interpretations can be formulated as well.

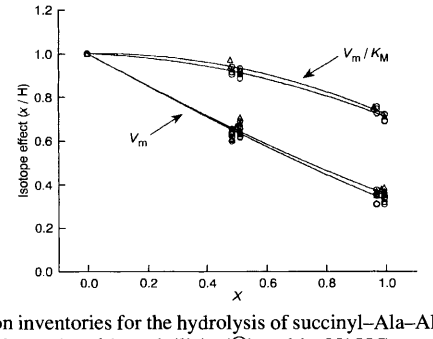

Fig. 1 Proton inventories for the hydrolysis of succinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide catalysed by subtilisin (○) and its N155G mutant (△). For the V_m/K_M inventories the upper and lower lines are best-fit curves for wild-type and mutant enzymes, respectively, whereas for the V_m inventories this order is reversed; x = atom fraction of deuterium in the solvent.

It was pointed out by one of us[12] some time ago that the very large number of exchangeable hydrogens in the enzymes and their aqueous solvation shells confuses mechanistic interpretation of proton inventories on enzyme-catalysed reactions. The present results would seem to support that allegation by casting further doubt upon the ability of proton inventories to enumerate protons undergoing transfer in serine protease-catalysed reactions.

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Footnotes

† This relationship is often called the Gross–Butler equation, after the pioneering work done by Gross[13] and Butler[14] on solvent isotope effects in H_2O–D_2O mixtures. That, however, is a misnomer, for this relationship does not appear in any of Gross' or Butler's publications. Gross and Butler derived a special case of eqn. (1), that pertaining to the ionization of a monobasic acid; the completely general relationship of eqn. (1), which is applicable to any protonic process, first appeared in ref. 4.

‡ Hydrogen-bonding interactions in the oxyanion hole of subtilisin are provided by Thr-220 and Ser-221 in addition to Asn-155. Site-directed mutagenesis has shown, however, that the effect of Thr-220 is less than half that of Asn-155.[7c] The stabilization provided by Ser-221 cannot be assessed in the same way because the hydrogen-bond donor here is a main-chain amide group, but the fact that this interaction operates at a greater distance from the substrate than does that of Asn-155[7c] suggests that it also contributes only a small fraction of the total oxyanion hole effect.

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