Direct Measurement of the Uncatalyzed Rate of Hydrolysis of a Peptide Bond

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A quantitative estimation of an enzyme's efficiency can be calculated once the rate constant for the analogous uncatalyzed reaction has been determined.[1−3] Recently, in the discussion of their study of orotidine 5′-phosphate decarboxylase, Radzicka and Wolfenden[4] compiled a list of enzymes for which the "rate enhancement" (k_{cat}/k_{uncat}) and the "catalytic proficiency" [(k_{cat}/K_m)/k_{uncat}] have been calculated. This list is remarkable for its brevity, a fact due to the difficulty of measuring the corresponding uncatalyzed rate of most enzyme-catalyzed reactions. Furthermore, for those enzymes with the highest efficiencies, the corresponding uncatalyzed rate has typically been extrapolated from data obtained at high temperature and/or extreme pH.

Here, we report the uncatalyzed rate constant for the hydrolysis of the carboxypeptidase A substrate, hippurylphenylalanine,[5,6] in 25 mM borate buffer/0.5 M NaCl at pH 9 and 25 °C, conditions identical to those utilized in the enzymatic reaction. (Carboxypeptidase A was chosen because its mechanism of action involves direct attack of water upon the peptide bond.[7]) Nearly a decade ago, Kahne and Still[8] reported the first measurement of peptide-bond hydrolysis at neutral pH, and this value was in fact used by Radzicka and Wolfenden to calculate the efficiency of carboxypeptidase A. However, the uncatalyzed rate constant was measured using a heterogeneous system involving a tetrapeptide covalently linked to a polyacrylamide resin,[8] while the enzymatic kinetic constants to which it was compared[9] were determined using a different substrate under different assay conditions.

The assay employed here involves the derivatization of the free amino functionality liberated upon peptide bond hydrolysis with naphthalenedialdehyde in the presence of cyanide (NDA/CN), reagents previously described by de Montigny *et al.*[10] With NDA/CN as little as 200 fmol of an amino acid can be detected.[11,12] The resulting 1-cyano-2-substituted-benz[*f*]isoindole (CBI) derivatives are more stable and have a higher fluorescence intensity than those obtained upon reaction with *o*-phthalaldehyde/2-mercaptoethanol (OPA/2-ME), a reagent commonly used in amino acid analyses.[10] We have used the NDA/CN assay to measure the rate of hydrolysis of hippurylphenylalanine (Figure 1a). At various reaction times, aliquots were treated with NDA/CN, and the CBI-derivative of the product phenylalanine (Figure 1b) was detected by reverse-phase HPLC.

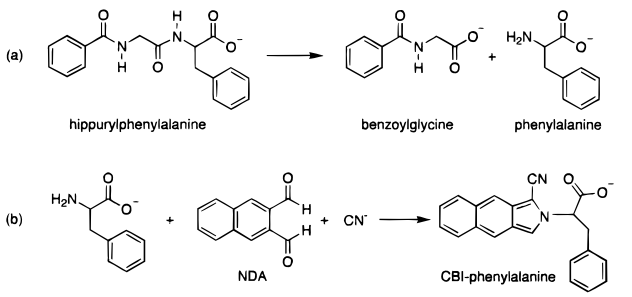

Figure 1. (a) Hydrolysis of hippurylphenylalanine. (b) Reaction of phenylalanine with NDA/CN.

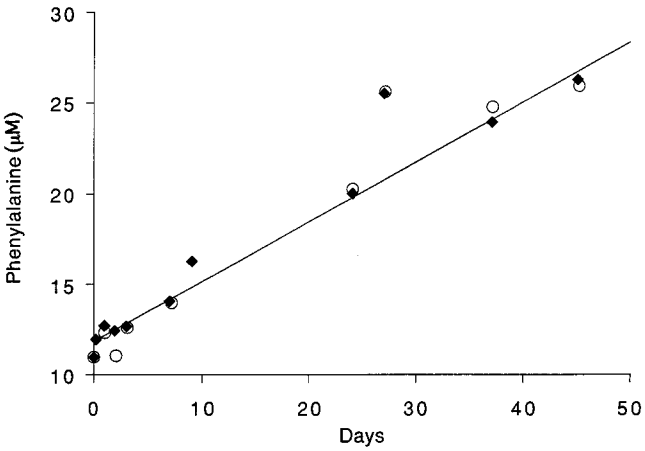

Figure 2. Appearance of phenylalanine, detected after derivatization with NDA/CN, during the uncatalyzed hydrolysis of 30 mM hippurylphenylalanine at pH 9. Open circles and solid diamonds indicate data from duplicate incubations. The phenylalanine present at time zero corresponded to 0.04% of the total substrate and could not be removed.

The NDA/CN assay allowed the uncatalyzed hydrolysis of 30 mM hippurylphenylalanine to be followed under conditions identical to those used for the carboxypeptidase A-catalyzed hydrolysis (Figure 2).[13] A value of 1.3 (±0.1) × 10^{-10} s^{-1} was determined for k_{uncat}, which yields a half-life of 168 years for hydrolysis in the absence of enzyme.[14] The final amount of phenylalanine detected at the end of the 50-day incubation corresponded to approximately 0.09% hydrolysis of the substrate. Hydrolysis of the benzoylglycine bond of hippurylphenylalanine (Figure 1a) was also apparent, but at such a small rate than an accurate measure was unobtainable. To experimentally establish the kinetic order in peptide concentration,

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(13) A solution of hippurylphenylalanine (30 or 3.0 mM) in 1.2 mL of 25 mM borate buffer/0.5 M NaCl, pH 9.0, was passed through a 0.2 μm filter to remove contaminants and incubated at 25 °C. Periodically 35 μL aliquots were removed, and 35 μL of 5 μM leucine in 0.1 M borate buffer, pH 9.3, (the internal standard), 20 μL of 0.01 M NDA in methanol, and 10 μL of aqueous 0.05 M NaCN were added. The reaction mixture was allowed to stand for 15 min at room temperature, at which time 10 μL was analyzed by HPLC. Alltech Econosphere C_{18} 5U column (150 × 4.6 mm) was eluted with a gradient of 50:50−100:0 MeOH:H_2O (0.05% TFA) over 50 min: λ_{ex} = 246 nm and λ_{em} = 490 nm. The concentration of CBI-phenylalanine was determined by comparison to the internal standard CBI-leucine as referenced to a standard curve. ^{1}H-NMR spectra of hippurylphenylalanine at concentrations ranging from 0.1875 to 45 mM in deuterated buffer (25 mM borate buffer/0.5 M NaCl) at pH 9 showed no increase in line width, a result indicative of a lack of peptide aggregation.

(14) Values of k_{uncat}, and the standard error, were determined directly from the integrated first-order rate equation using the program Microsoft Excel 3.0. Values of K_m and V_{max}, and the standard errors, were determined using the program HYPER as described by Cleland.[15]

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the uncatalyzed hydrolysis of 3 mM hippurylphenylalanine was followed. The data, again collected over 50 days, yielded a k_{uncat} of 8.2 ($\pm$1.9) $\times$ 10^{-11} s^{-1} (the larger experimental error is due to the extremely small amounts of CBI-phenylalanine present).[14] The calculation of essentially identical first-order rate constants from the 30 and 3 mM incubations of hippurylphenylalanine indicates that the hydrolysis reaction is indeed first order in peptide.

Incubations of 6 mM hippurylphenylalanine at pH 7 (25 mM Hepes/0.5 M NaCl) showed no detectable hydrolysis after 50 days, indicative that hydrolysis is slower at neutral pH than at pH 9. At pH 11 (25 mM triethylamine/0.5 M NaCl) a k_{uncat} of 2.7 ($\pm$0.2) $\times$ 10^{-10} s^{-1} was obtained with 3 mM hippurylphenylalanine.[16] (The low solubility of the substrate at pH 7 and 11 precluded the use of higher concentrations.) This two-fold increase in rate upon increasing the pH from 9 to 11 is consistent with the earlier results obtained by Kahne and Still[8] and with studies from our lab on the hydrolysis of phenylacetylglycyl-D-valine.[17]

The rate of the carboxypeptidase A-catalyzed hydrolysis of hippurylphenylalanine at pH 9 and 25 °C was also measured using the NDA/CN assay.[18] The kinetic constants determined (k_{cat} = 61 $\pm$ 4 s^{-1}, K_m = 0.29 $\pm$ 0.06 mM) were comparable to those obtained with a standard spectrophotometric assay[14,19] (k_{cat} = 61 $\pm$ 8 s^{-1}, K_m = 0.36 $\pm$ 0.09 mM), thereby confirming the applicability of the NDA/CN assay as an accurate method for acquiring kinetic data (Figure 3). The catalyzed and uncatalyzed kinetic values ascertained using the NDA/CN assay can be directly compared and give a rate enhancement (k_{cat}/k_{uncat}) of 4.7 $\times$ 10^{11}-fold and a catalytic proficiency [(k_{cat}/K_m)/k_{uncat}] of 1.6 $\times$ 10^{15} M^{-1} for carboxypeptidase A.

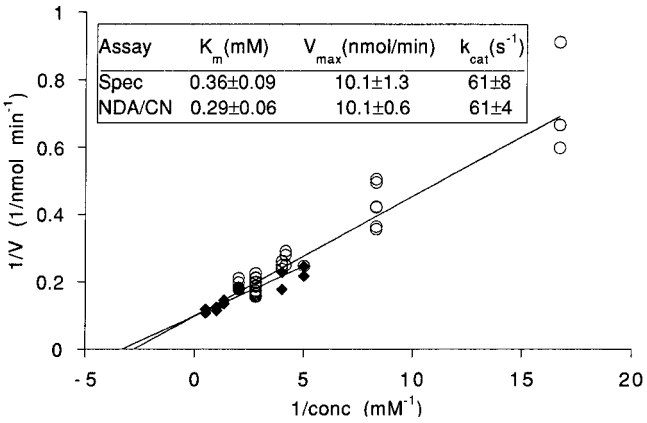

Figure 3. Lineweaver-Burk plot of carboxypeptidase-catalyzed hydrolysis of hippurylphenylalanine monitored spectrophotometrically (open circles) and with NDA/CN (solid diamonds).

In summary, the rate enhancement and catalytic proficiency of carboxypeptidase A have been directly calculated from data obtained under identical reaction conditions. Moreover, the NDA/CN assay employed provides, in principle, a general method for the determination of the uncatalyzed rates of hydrolysis of peptide bonds (with the exception of those with a carboxy terminal prolyl residue) without the use of extreme temperature or pH.

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Supporting Information Available: Representative HPLC chromatograms showing the appearance of phenylalanine, detected after derivitization with NDA/CN, during the uncatalyzed hydrolysis of 30 mM hippurylphenylalanine (pH 9); ^{1}H-NMR spectra of hippurylphenylalanine (pH 9) at concentrations ranging from 0.1875 to 45 mM (9 pages). Ordering information is given on any current masthead page.

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(16) Incubations were similar to those described above[13] except that 25 mM triethylamine/0.5 M NaCl, pH 11 buffer (by the end of the 27 day incubation, the pH had dropped to 10.6), and an elution gradient of 65:35−85:15 MeOH:H_2O (0.05% TFA) over 30 min were used.

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(18) A solution of hippurylphenylalanine (0.20, 0.25, 0.50, 0.75, 1.0, or 2.0 mM) in 3.6 mL of 25 mM borate buffer/0.5 M NaCl, pH 9.0, was thermally equilibrated at 25 °C. Carboxypeptidase (0.44 μg) in 40 μL of the borate buffer was then added. After the addition of enzyme, 150 μL aliquots were removed at 1 min intervals, and 3 μL of 35% perchloric acid was added to each aliquot. (An aliquot was also analyzed prior to the addition of enzyme.) To 25 μL of the quenched sample were added 25 μL of 50 μM leucine in H_2O, 20 μL each of 0.1 M borate buffer (pH 12.3) and 0.01 M NDA in methanol, and 10 μL of aqueous 0.05 M NaCN. After 15 min at room temperature, 10 μL of the reaction mixture was analyzed by HPLC as described above,[13] except using an elution gradient of 50:50−75:25 CH_3CN:H_2O (containing 0.05% TFA) over 20 min. All data points were obtained in duplicate and were corrected for loss of enzymatic activity over time.

(19) Incubations were similar to those described above[18] with the exceptions that a total reaction volume of 1 mL and initial hippurylphenylalanine concentrations of 0.06, 0.12, 0.24, 0.25, and 0.36 mM were employed. The progress of the reaction was monitored at 254 nm. ($\Delta\epsilon$ = 280 M^{-1} cm^{-1} for hippurylphenylalanine.[6])