Mechanistic Studies of Carboxypeptidase Y from *Saccharomyces cerevisiae*

pH AND pD PROFILES AND INACTIVATION AT LOW pH (pD) VALUES

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Steady-state kinetics of carboxypeptidase Y, a proteinase from yeast, were studied by using the reaction of 4-nitrophenyl trimethylacetate as a probe. The pH profile of k_{cat} is sigmoidal in H₂O-based buffers for the carboxypeptidase Y-catalysed hydrolysis of this ester (k_{cat} , referring to the rate of deacylation of trimethylacetyl-carboxypeptidase Y). The corresponding pD profile in ²H₂O is doubly sigmoidal, with inflexions at pD ~3.8 and ~6.8. The ionization of $\text{p}K_{\text{app}} \sim 3.8$ is caused by a rapid inactivation in ²H₂O media by a process that is only slowly reversed on transfer to pH 7.00 phosphate buffer in H₂O. The corresponding inactivation in H₂O-based buffers of low pH is considerably slower (~30-fold), follows a first-order rate-dependence and is very strongly pH-dependent, indicating some form of co-operative change in enzyme tertiary structure.

The yeast proteinase carboxypeptidase Y (EC 3.4.16.1) is a glycoenzyme, isolatable in distinct molecular forms, differing in their catalytic properties and in the amounts of attached sugars (Margolis *et al.*, 1978). It exhibits apparent similarities to both the serine and cysteine proteinases, on the one hand, and to the pancreatic metallo-carboxypeptidases, on the other. Carboxypeptidase Y is a relatively non-specific single-chain exopeptidase with no metal-ion-dependence (Hayashi *et al.*, 1973, 1975) and no intrinsic endopeptidase activity (Hermansen *et al.*, 1972; Hayashi *et al.*, 1973; Lee & Riordan, 1978), capable of cleaving even proline residues from proteins. Its high thermal stability, apparent resistance to pH extrema and activity in 6M-urea or in sodium dodecyl sulphate media have led to its increasing use in protein sequencing and structural studies (Hayashi, 1977; Liberatore *et al.*, 1976; Martin *et al.*, 1977).

With aryl trimethylacetates, 'burst kinetics' ($[S]_0 \gg [E]_0$) have been observed and studied in detail by using stopped-flow techniques (Douglas *et al.*, 1976). The results were quantitatively consistent with formation of a physical Michaelis complex ES (dissociation constant K_s) followed by a covalent change (rate constant k_2) leading to an acyl-enzyme, ES', and the phenolate component of the products. Deacylation (rate constant k_3) of trimethylacetyl-

carboxypeptidase Y then leads to free enzyme and trimethylacetate ion (eqn. 1):

Although it was recognized that biphasic kinetics of the type observed merely indicate a change in rate-determining step, there is some additional support for the acyl-enzyme mechanism. Thus values of k_{cat} for a series of aryl-substituted trimethylacetates with carboxypeptidase Y are independent of the leaving group (Douglas *et al.*, 1976), in contrast with the rate constants for attack of OH⁻ ion and imidazole on these esters (Douglas *et al.*, 1977). Under single-turnover conditions ($[E]_0 > [S]_0$), by using a stopped-flow technique with 4-nitrophenyl cinnamate as substrate, the 4-nitrophenoxide ion product is released previously to, and faster than, the cinnamate ion, as predicted from eqn. (1) (Douglas *et al.*, 1975). In addition, an intermediate (as yet unisolated) was detected kinetically in the enzymic hydrolysis of 4-methoxyphenyl cinnamate, presumably cinnamoyl-carboxypeptidase Y (Douglas *et al.*, 1975).

Although such observations are reminiscent of α -chymotrypsin, deacylation of trimethylacetyl- α -chymotrypsin depends, crucially, on the state of ionization of a functional group of $\text{p}K_{\text{app}} = 6.7$ (Bender *et al.*, 1962). Deacylation of trimethyl-

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acetyl-carboxypeptidase Y, in contrast, shows a non-crucial sigmoidal pH-dependence, with $pK_{app.} \sim 5.1$. The rate constant at low pH is 41% of the maximal rate constant in alkaline media (Douglas *et al.*, 1976). The group of $pK_{app.} \sim 5.1$ may be a carboxy group or a perturbed histidine residue.

Other studies directed towards detection of acyl-enzyme intermediacy have been reported (Bai *et al.*, 1975). For example, in the carboxypeptidase Y-catalysed cleavages of *N*-acetyl-L-tryptophan derivatives it was stated that values of $k_{cat.}$ were nearly constant, indicating that k_3 was rate-determining (Bai *et al.*, 1975). In addition, studies of the solvent deuterium isotope effect have been reported (Bai *et al.*, 1975). These were performed at a single pH (pD) and for derivatives whose K_m and $k_{cat.}$ values may well be complex, as eqn. (1) yields $k_{cat.} = k_2 k_3 / (k_2 + k_3)$ and $K_m = K_s k_3 / (k_2 + k_3)$ on kinetic analysis. In addition, the inhibition pattern exhibited by L-phenylalanine apparently changes on going from H_2O to 2H_2O media in studies of *N*-acetyl-L-phenylalanyl ethyl ester.

Consequently, we have studied the solvent deuterium kinetic isotope effect [over a wide pH (pD) range] on the carboxypeptidase Y-catalysed hydrolysis of 4-nitrophenyl trimethylacetate, for which detailed knowledge of the microscopic rate constants in H_2O had already been obtained.

Materials

Compressed baker's yeast was obtained from Anheuser-Busch (Pittsburgh, PA, U.S.A.), DEAE-cellulose DE-52 from Whatman (Maidstone, Kent, U.K.) and Sephadex A-50 from Pharmacia Fine Chemicals (Uppsala, Sweden). *N*-Acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt) and *N*-benzyloxycarbonyl-L-glutamyl-L-tyrosine (Cbz-Glu-Tyr) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 4-Nitrophenyl trimethylacetate, from a previous study, was recrystallized from benzene. Acetonitrile (Pesticide grade; Fisher Chemical Co., Silver Spring, MD, U.S.A.) was further purified by molecular-sieve (Linde 4A) treatment, followed by distillation from a small quantity of P_2O_5 and finally distillation off CaH_2 . 2H_2O (99.8 atom% 2H ; Aldrich Chemical Co., Milwaukee, WI, U.S.A.) was either used as received or redistilled off $KMnO_4$ for further purification. Buffer solutions were prepared from reagent-grade materials.

Carboxypeptidase Y was purified by the procedure of Hayashi *et al.* (1973), with slight modifications. Sodium dodecyl sulphate/polyacrylamide-gel rod electrophoresis was performed with a Pharmacia GE-II apparatus, by the method of Weber & Osborn (1969). Protein samples (approx. 1 mg/ml) were boiled with 0.1% sodium dodecyl

sulphate/1% dithiothreitol (Sigma) for 10 min on a water bath; less drastic treatment does not completely unfold carboxypeptidase Y, leading to multiple bands on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Reservoir buffer was 0.1 M-potassium phosphate buffer, pH 7.3, containing 0.5% sodium dodecyl sulphate. Electrophoresis was performed for 4 h at constant current (1.8 mA/rod).

Methods

Absorption coefficients on H_2O and 2H_2O

Around neutrality the absorption change of the hydrolysis reaction of 4-nitrophenyl trimethylacetate is very pH-sensitive, as the pK_a of 4-nitrophenol is approx. 7. Consequently, for any given pH or pD, the absorption change was measured. Complete hydrolysis of substrate in 10 mM-NaOH (30 min) was performed. The absorbances of samples of this hydrolysate in the appropriate buffer medium allowed calculation of the absorption change. Above pH (or pD) ~ 6.4 , kinetics of 4-nitrophenyl ester hydrolysis were studied at 400 nm, and below pH ~ 6.4 at 340 nm.

Enzyme concentrations and assays

Approximate enzyme concentrations were measured by using $A_{280}^{1\%} = 15$ and a molecular weight of 61000 (Aibara *et al.*, 1971; Margolis *et al.*, 1978). Accurate functional enzyme concentrations were determined by active-site titration as described elsewhere (Margolis *et al.*, 1978).

Titrimetric measurement of the Ac-Tyr-OEt activity of carboxypeptidase Y was performed at 25°C at pH 8.00 (10 mM-Ac-Tyr-OEt in 0.1 M-KCl) as a standard assay. One unit of Ac-Tyr-OEt activity is 1 μ mol of Ac-Tyr-OEt hydrolysed/min.

The peptidase activity could be studied either by the colorimetric ninhydrin method of Hayashi *et al.* (1973) (for the determination of liberated tyrosine) or, quantitatively, by the decrease in absorbance at 224 nm (Kuhn *et al.*, 1974), determined to be the best wavelength by repetitive spectral scanning of a reacting mixture.

In the ninhydrin method, enzyme solution (0.1 ml) and substrate (0.1 ml of 3 mM-Cbz-Glu-Tyr) were added to 0.8 ml of 50 mM-sodium acetate buffer, pH 5.5, in a test tube. After incubation at 25°C for 15 min, reaction was terminated by addition of ninhydrin reagent and heating in a boiling-water bath. Blanks with substrate and enzyme absent in turn were performed.

Kinetic procedures

Spectrophotometric studies were performed with a GCA-McPherson 707-K double-beam spectrophotometer, whose cuvette chamber was thermo-

statically controlled at $25.00 \pm 0.02^\circ\text{C}$ by means of a Haake E52 thermoregulator pump.

The Radiometer recording pH-stat and titration system used consisted of a PHM 64 pH-meter (0.001 pH readability) with a TTT60 Titrator and REC 61 chart recorder. The glass reaction vessel was kept at $25.00 \pm 0.02^\circ\text{C}$ by means of a Thermomix model 1420. A type ABU 12 autoburette (0.25 ml capacity) delivered titrant (50 mM-NaOH). All pH-stat reactions were carried out under a stream of scrubbed CO_2 -free dry N_2 . The pH-meter was standardized against Fisher standard buffers; titrant concentration was checked against standard acid.

Generally, pH values measured before and after reaction differed by <0.04 pH unit; post-reaction values were used in calculations. As the maximum amount of acetonitrile was low (never greater than 4%, v/v) it was assumed (Keszdy & Bender, 1962) that this had a negligible effect on the electrode reading.

Studies with $^2\text{H}_2\text{O}$

The apparent pH values of 1.2 mM-HCl in H_2O and 1.2 mM- ^2HCl in $^2\text{H}_2\text{O}$ were measured, and the relationship $\text{pD} = \text{pH}_{\text{meas.}} + 0.353$ was established for the pH-meter used throughout.

For studies of inactivation in $^2\text{H}_2\text{O}$, carboxypeptidase Y was incubated in buffers of various pD values at ice-bath temperature for 15 min, in the presence and absence of substrate. The (remaining) activity of incubated enzyme was measured in an appropriate buffer.

Results

pH and pD profiles of $k_{\text{cat.}}$ for 4-nitrophenyl trimethylacetate

By using the procedure previously detailed (Douglas *et al.*, 1976), values of $k_{\text{cat.}}$ ($=k_3$) for 'Anheuser-Busch carboxypeptidase Y' were obtained for the pH range 3–8. Each point represented, in effect, an active-site titration and $k_{\text{cat.}}$ was taken as the zero-order slope rate (steady state) divided by the initial burst (proportional to the functioning enzyme concentration). The data are presented in Fig. 1, along with the values of k_3 from a previously published study (Douglas *et al.*, 1976) for 'Fleischmann carboxypeptidase Y', i.e. the enzyme prepared in the same manner, but from Fleischmann's baker's yeast. There is no significant difference in deacylation rate or its sensitivity towards pH changes for these preparations, in agreement with the previous study.

A similar experiment, but with enzyme from Anheuser-Busch yeast only, was carried out to determine the pD-dependence of $k_{\text{cat.}}$ in $^2\text{H}_2\text{O}$ for 4-nitrophenyl trimethylacetate. The results are col-

lected in Table 1 and shown in Fig. 2. The pD-dependence of $k_{\text{cat.}}$ is doubly sigmoidal, with $\text{p}K_{\text{app.}}^{\text{D}}$ values of ~ 3.8 and ~ 6.8 and a limiting value of $k_{\text{cat.}}$ at high pD of $8.15 \times 10^{-3} \text{ s}^{-1}$. The con-

Fig. 1. Steady-state pH profile (k_3) for the carboxypeptidase Y-catalysed hydrolysis of 4-nitrophenyl trimethylacetate at 25.0°C at 1.0 l.

Each value of k_3 was determined from the zero-order slopes at two different enzyme concentrations. All measurements were made with a single enzyme preparation from either Anheuser-Busch yeast (O) or Fleischmann yeast (□). Points are experimental; the continuous line is theoretical, obtained by using the Henderson-Hasselbach equation with $\text{p}K_{\text{app.}} = 5.35$ and limiting values of k_3 in acid of $1.00 \times 10^{-2} \text{ s}^{-1}$ and in base $2.50 \times 10^{-2} \text{ s}^{-1}$. Data for the Fleischmann-yeast enzyme were taken from Douglas *et al.* (1966). See the text for experimental details.

Table 1. Steady-state pD-profile data for carboxypeptidase Y-catalysed hydrolysis of 4-nitrophenyl trimethylacetate

Experimental details are given in the text. $k_{\text{cat.}}$ values were obtained from the zero-order (steady-state) slope at two different enzyme concentrations. pD values were from the relationship $\text{pD} = \text{pH}_{\text{meas.}} + 0.35$.

pD	$10^3 \times k_{\text{cat.}} (\text{s}^{-1})$
8.65	8.83
8.39	8.67
7.75	8.80
7.69	8.42
7.08	8.23
6.81	6.50
6.44	5.89
5.52	5.50
5.08	5.32
4.79	5.14
4.54	4.73
4.25	3.90
3.85	2.73
3.67	2.14
3.62	1.85
3.18	0.47
2.93	Not detected

Inactivation of carboxypeptidase Y at low pH

A corresponding inactivation occurs at low pH, but considerably more slowly, the activity decreasing with time until it reaches a steady value, which we have called $(k_{\text{cat.}})'_{\text{H}}$. A typical example, at pH 2.86, is shown in Fig. 4. The rates of inactivation in 0.05 M- and 0.10 M-sodium formate buffer are essentially the same, indicating that the inactivation is not a buffer effect. Inactivation can be analysed as a first-order kinetic process (see Fig. 4 for the fit to the first-order rate equation). Below pH 2.86 the rate of inactivation became high, and above pH 3.7 inactivation was extremely slow. Rates of inactivation were determined in the intermediate pH range, rate constants for inactivation being obtained from the best fit by non-linear regression analysis to a first-order rate equation. Data are collected in Table 3. The rate of inactivation is

Fig. 4. Time course of the inactivation of carboxypeptidase Y in H_2O -based formate buffers at pH 2.86. Values of $(k_{\text{cat.}})'$ were obtained by assaying samples in the 4-nitrophenyl trimethylacetate reaction in pH 7.00 H_2O -based phosphate buffer. Points are experimental (●, 0.1 M-formate buffer; ■, 50 mM-formate buffer). The continuous line is theoretical for a first-order reaction with a rate constant $(k_{\text{inact.}})$ of $1.98 \times 10^{-2} \text{ min}^{-1}$ and a limiting value of $(k_{\text{cat.}})'_{\text{H}}$ of $0.422 \times 10^{-1} \text{ s}^{-1}$. See the text for experimental details.

Table 3. Rates of inactivation of carboxypeptidase Y towards 4-nitrophenyl trimethylacetate in H_2O -based buffers at 0°C

Values of $k_{\text{inact.}}$ and $(k_{\text{cat.}})'_{\infty}$ were obtained by least-squares regression analysis of the data to fit a first-order rate equation. The control (pH 7.00) retained activity at $2.45 \times 10^{-2} \text{ s}^{-1}$ ($k_{\text{cat.}}$ for 4-nitrophenyl trimethylacetate) throughout the experiments. $k_{\text{inact.}}$ values were determined by incubation of a sample of carboxypeptidase Y in the appropriate buffer in an ice bath. Samples were withdrawn at suitable times and the remaining enzyme activity was assayed by determining $k_{\text{cat.}}$ for 4-nitrophenyl trimethylacetate at pH 7.00. Experimental details are given in the text.

pH	$k_{\text{inact.}}$ (h^{-1})	$10^2 \times (k_{\text{cat.}})'_{\infty}$ (s^{-1})
2.25	$t_1 < 30 \text{ s}$	0.117
2.86	1.19	0.422
3.00	0.408	0.373
3.27	0.257	0.909
3.30	0.0845	0.987
3.50	0.0527	0.510*
3.66	0.00825	0.253*
3.90	Not determined	2.30†
4.40	Not determined	2.39‡

* Only small changes in the activity were observed over 24 h, requiring a large extrapolation to obtain $(k_{\text{cat.}})'_{\infty}$ and $k_{\text{inact.}}$, with consequent loss of precision. For this reason these points are excluded from Fig. 5.

† This was the value of $(k_{\text{cat.}})'$ measured after 72 h.

‡ This was the value of $(k_{\text{cat.}})'$ measured after 48 h.

Fig. 5. pH profile of limiting values of $(k_{\text{cat.}})'_{\text{H}}$ reached after incubation of carboxypeptidase Y in various H_2O -based buffers

The $(k_{\text{cat.}})'_{\infty}$ values were obtained by non-linear regression analysis by using the first-order rate equation as described in the text. The line describes the ionization of $\text{p}K_{\text{app.}} = 3.4$. See the text for experimental details.

extremely pH-sensitive for this small pH range, and can be described by the linear relationship (eqn. 3):

$$\log(k_{\text{inact.}}) = 7.14 (\pm 0.11) - 2.46 (\pm 0.34) \text{ pH} \quad (3)$$

In general, the activity remaining did not reach zero but levelled off (e.g. see Fig. 4). The 'equilibrium' values of $(k_{\text{cat.}})'_{\text{H}}$ after incubation, $(k_{\text{cat.}})'_{\infty}$, in various H_2O -based buffers were obtained from the fit to the first-order equation $(k_{\text{cat.}})'_{\text{H}} = (k_{\text{cat.}})'_{\infty} e^{-k_{\text{H}}t}$ and are plotted versus pH in Fig. 5. Although the scatter is rather high, for visualization purposes we have drawn an ionization curve for an acid of $\text{p}K_{\text{app.}} 3.4$ through the data.

Incubation in pH 3.0 H_2O -based buffer also destroys activity towards Cbz-Glu-Tyr. Although only a few points were determined for the time course with Ac-Tyr-OEt, the overall course was similar to that with the $k_{\text{cat.}}$ of 4-nitrophenyl trimethylacetate as the assay for activity. Incubation of carboxypeptidase Y in buffers of pH 8.0, pD 8.4, pH 3.0 and pD 3.0 gave the relative peptidase activities of 1.0, 1.0, 0.48 and 0 respectively.

Discussion

The difference between the doubly sigmoidal $k_{\text{cat.}}$ (k_3) profile in $^2\text{H}_2\text{O}$ and the singly sigmoidal k_3 profile in H_2O for the carboxypeptidase Y-catalysed hydrolysis of 4-nitrophenyl trimethylacetate can be explained by the inactivation at low pH and pD. The rate of inactivation at low pH is sufficiently slow to be undetected on the time scale of the experiments to measure the k_3 -pH profile. However, deactivation at low pD occurs in a few seconds, i.e. faster than the time taken to measure $k_{\text{cat.}}$ in $^2\text{H}_2\text{O}$. The limb of $\text{p}K_{\text{app.}}^{\text{D}} \sim 3.8$ refers to the inactivation process in acidic $^2\text{H}_2\text{O}$ media. The higher $\text{p}K_{\text{app.}}^{\text{D}}$ (~ 6.8) presumably corresponds to the ionization of $\text{p}K_{\text{app.}}^{\text{H}} = 5.35$ observed in H_2O . However, the shift in $\text{p}K_{\text{a}}$ expected of an acid of $\text{p}K_{\text{a}}^{\text{H}} \sim 5$ on transfer from H_2O to $^2\text{H}_2\text{O}$ is only of the order of +0.5 pK unit (Bell & Kuhn, 1963). It is likely that $k_{\text{cat.}}$ in $^2\text{H}_2\text{O}$ is still equal or close to k_3 , in view of the retention of quantitative bursts. The unusually large shift (of $\sim +1.5$ pK units) for the enzyme may indicate a change in environment for the feature responsible for the ionization with $\text{p}K_{\text{app.}}^{\text{H}} \sim 5.3$, or an entirely different ionization may be involved. It has been reported (Bai *et al.*, 1975) that, in the carboxypeptidase Y-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl ethyl ester, inhibition by L-phenylalanine was non-competitive in $^2\text{H}_2\text{O}$ (pD 7.0), but competitive in H_2O (pH 6.5). This change in inhibition type may indicate a difference in environment (e.g. a minor conformational change or a configurational alteration of the active site), such as that discussed above.

There is a report (Bai *et al.*, 1975) of $k_{\text{cat.}}$ ($\text{H}/^2\text{H}$)

equal to 2.33 and 1.34 for carboxypeptidase Y-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl ethyl ester and benzyloxycarbonyl-L-phenylalanyl-L-leucine respectively in basic media. One can calculate a kinetic solvent deuterium isotope effect on k_3 ($\text{H}/^2\text{H}$) in basic media from our results of ~ 2.9 for hydrolysis of trimethylacetyl-carboxypeptidase Y (by using the alkaline plateau rate constants for comparison). For the low-pH plateaux [comparing values of $1 \times 10^{-2} \text{ s}^{-1}$ for k_3 in H_2O (see Fig. 1) and $5.4 \times 10^{-3} \text{ s}^{-1}$ for k_3 in H_2O (see Fig. 2)] a lower value of 1.9 is obtained. This change in kinetic solvent isotope effect may reflect the effect of the same feature as perturbs the value of $\text{p}K_{\text{app.}}$, described above. With such possibilities of conformational or even mechanistic changes with pH, it is difficult to use kinetic solvent isotope effects on $k_{\text{cat.}}$ as a mechanistic probe for this enzyme. This is true even of the simple kinetic system chosen here (with $k_{\text{cat.}} = k_3$, a single microscopic rate parameter) and underscores the difficulties of interpreting kinetic solvent isotope effect data on undefined $k_{\text{cat.}}$ values, which may well be composite, for more complex hydrolyses.

The inactivation at low pH probably explains the observation (Hayashi *et al.*, 1968) that, in the preparation of carboxypeptidase Y from Oriental yeast, there was a rapid appearance of activity from a crude yeast autolysate at pH 3, but that this was followed by a decrease in activity. Hayashi (1976) has reported that the salt-free pure enzyme loses some 20% of its activity on being freeze-dried, but that almost full activity is restored by leaving a solution of the freeze-dried enzyme overnight at 5°C or at -20°C . In addition, freeze-dried enzyme when redissolved requires a long equilibration time (~ 24 h) before a stable reading of the A_{280} can be made (Margolis *et al.*, 1978). These observations may again reflect conformational flexibility in it. It is also apposite to report that this enzyme has been isolated in several distinct molecular forms, differing primarily in attached carbohydrate. These differences are reflected in the microscopic pre-steady-state rate parameters for the 4-nitrophenyl trimethylacetate reaction as well as in the c.d. spectra (Margolis *et al.*, 1978).

The rates of inactivation at low pH and low pD are enigmatic; e.g. consider the tremendous difference in the rates of deactivation. In $^2\text{H}_2\text{O}$ at pD 3.0, over 80% of the activity is lost in the first minute; in H_2O at pH 2.86 (Fig. 4) the half-life is approx. 35 min ($= 0.7/k_{\text{inact.}}$). Such an enormous inverse apparent kinetic solvent isotope effect of over 30 ($^2\text{H}/\text{H}$) is untypical of a simple single-step process, and presumably indicates some form of co-operative breakdown of catalytically viable tertiary structure as an explanation of the inactivation process. The rate law for the dependence of rate of inactivation on

pH (eqn. 3) with its high coefficient for pH-dependence again indicates a complex inactivation pathway. Specific acid catalysis would produce a coefficient of -1.0 .

The dangers of $^2\text{H}_2\text{O}$ solvent perturbations in protein chemistry have long lurked as a caveat to kinetic solvent isotope studies, to protein n.m.r. in $^2\text{H}_2\text{O}$ and, more recently, to neutron-scattering studies of biological materials. Although the H_2O - $^2\text{H}_2\text{O}$ 'solvent' change can alter protein conformation for ribonuclease (Hermans & Scheraga, 1959; French & Hammes, 1965) as well as possibly altering the degree of association of some oligomeric proteins (Henderson *et al.*, 1970) and affecting proline racemase (Cardinale & Abeles, 1968), this major kinetic difference between the acidic inactivation of carboxypeptidase Y in H_2O and $^2\text{H}_2\text{O}$ contrasts sharply with the minor effects of the $^2\text{H}_2\text{O}$ - H_2O change for proteins in general. For example, α -chymotrypsin has been carefully studied in $^2\text{H}_2\text{O}$ media and no unusual effects were noted. Changes in the magnitudes of rate constants or pK values are in line with straightforward physico-chemical expectation (Bender & Hamilton, 1962). The results with carboxypeptidase Y underscore the need for careful control experiments with proteins in $^2\text{H}_2\text{O}$ media.

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