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Chemistry

Evidence for the general base mechanism in carboxypeptidase A-catalyzed reactions: Partitioning studies on nucleophiles and $H_2{}^{18}O$ kinetic isotope effects

(anhydride solvolysis/zinc complex)

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ABSTRACT **Methanol does not detectably compete with water in carboxypeptidase-catalyzed cleavage of any substrate, although it is preferentially reactive in a model for the proposed nucleophilic mechanism for the enzyme that involves an anhydride intermediate. To test for such a common intermediate in the cleavage of related peptide and ester substrates, a method has been developed to examine $H_2{}^{16}O$–$H_2{}^{18}O$ kinetic isotope-partitioning effects. The finding that benzoylglycylphenylalanine has an isotope effect of 1.019 ± 0.002 while benzoylglycyl-β-L-phenyllactate shows a small inverse isotope effect excludes most versions of a nucleophilic mechanism having a common anhydride intermediate. The bulk of the available evidence strongly favors the previously proposed general base mechanism.**

Much attention has been devoted to studies of the mechanism by which carboxypeptidase catalyzes hydrolysis (1–9). In spite of this the overall mechanistic path is still not clear, to say nothing of the details of the path.

Three functional groups of the enzyme seem to play catalytic roles: a bound Zn^{2+}, the COO^- group of glutamate-270, and the phenolic OH of tyrosine-248. The tyrosine is apparently required for the cleavage of peptides but not for the cleavage of ester substrates of the enzyme (8). The Zn^{2+} can be replaced by other metal ions (1), with different effects on the catalytic ability of the enzyme to hydrolyze peptides and esters. In most mechanistic suggestions (9), the Zn^{2+} acts as a Lewis acid by coordinating with the carbonyl oxygen of the scissile group in the substrate, and the phenol assists protonation of the leaving group either by simple proton transfer or by acting as both donor and acceptor to facilitate an internal switch of protons in the tetrahedral intermediate (6). However, there is no universal agreement on this; Mock (10) has suggested a mechanism in which the tyrosine phenol group acts, in its ionized form, as a base rather than as an acid.

One of the major ambiguities concerns the role of the glutamate-270 COOH group. In the Mock mechanism, it acts as an acid, but in other mechanisms it functions, in its ionized form, as either a nucleophile or a general base. In nucleophilic mechanisms, it attacks the scissile carbonyl group to form an intermediate anhydride, an acyl–enzyme intermediate. In the general base mechanisms, glutamate-270 delivers a water molecule to the scissile carbonyl, forming a tetrahedral intermediate that decomposes to products without the intervention of any acylated enzyme. Chemical analogies exist for both mechanisms.

Nucleophilic attack on amides and esters to form anhydride intermediates is well precedented. An excellent example is found in the maleamic acids, in which an intermediate maleic anhydride is formed (11). In a recent further investigation (12) of a system investigated earlier (13), it was shown that this mechanism can be assisted with a phenolic group, in imitation of the combined role normally suggested for glutamate-270 and tyrosine-248 in enzyme-catalyzed amide cleavage. In contrast, a general base mechanism for assistance by a COO^- is found in the hydrolysis of aspirin (14), even though the nucleophilic mechanism would also have been available to this substance.

In chemical systems, the choice of one or the other mechanism seems to be dictated by whether the intermediate can be formed by a fragmentation or not. The maleamic acids or the corresponding maleate half-esters can form anhydride intermediates, converting stable amide or ester groups to enthalpically less-stable anhydride groups, because of the driving force associated with fragmentation of the substrate and the corresponding entropy increase associated with departure of the leaving group (15). In the case of aspirin, the anhydride intermediate would be formed by a rearrangement, rather than a fragmentation; without the entropy driving force of a fragmentation, this is apparently a less favorable path for hydrolysis than is the general base mechanism, which does not involve formation of a high-enthalpy anhydride intermediate.

Many hydrolytic enzymes use a two-step mechanism involving an acyl–enzyme intermediate. This mechanism is normally detectable by the observation of burst kinetics, indicating fragmentation with the departure of the substrate leaving group and by spectroscopic observation of the acyl–enzyme intermediate. No such evidence has yet been obtained for an intermediate in the hydrolysis of normal peptide and ester substrates by carboxypeptidase A. Furthermore, studies on ^{18}O exchange (6) exclude an anhydride mechanism for carboxypeptidase-catalyzed amide hydrolysis, except for a trapped-H_2O loophole considered below. However, Makinen, Kaiser, and co-workers (16, 17) have reported studies on a particular ester, *p*-chlorocinnamoyl-β-L-phenyllactate (**VI**). At low temperature, they see strong binding of the chromophore to the enzyme but no evidence for departure of the leaving group. Biphasic kinetics (which may simply involve a conformational change) are also observed. Based on these observations, they claim that an acyl–enzyme intermediate is being formed with this ester. Until there is direct evidence for an intermediate, this must certainly be considered a tentative conclusion. Even if it proves to be true, as pointed out (6), this is an unusual ester whose mechanism may not be typical. Normally, good substrate peptides and esters of related structure have comparable reactivities with the enzyme, but the peptide corresponding to ester **VI** is a poor substrate for the enzyme.

If all substrates are hydrolyzed by the enzyme through a mechanism involving an acyl–enzyme intermediate, then it should be possible to detect this common intermediate, regardless of origin, by its uniform further chemical behavior. Common intermediates from different substrates are fre-

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quently detected by observing identical partitioning when they are presented with a choice of nucleophiles, for instance. Such tests have been applied successfully with other hydrolytic enzymes that have demonstrably formed acyl–enzyme intermediates; in particular, many such intermediates preferentially react with MeOH rather than H_2O (6). Apparently the small MeOH molecule has no difficulty gaining access to the active sites of the enzymes examined, and it is a better nucleophile than H_2O toward many substrates. Thus, we decided to examine the MeOH–H_2O partitioning situation further with carboxypeptidase and with an appropriate model system. We have also examined a different partitioning, that between $H_2{}^{18}O$ and $H_2{}^{16}O$ in enzyme-catalyzed hydrolyses.

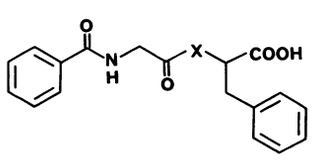
EXPERIMENTAL METHODS

Materials. Dimethylformamide was dried by distillation from CaH_2. Unenriched water was distilled and deionized. All organic solvents and buffer components were reagent grade or spectrograde and used without further purification. Labeled water ($H_2{}^{18}O$; 99%) was purchased from Stohler. The model anhydride (**I**) has been prepared previously in our laboratories. *p*-Chloro-*trans*-cinnamoyl-β-L-phenyllactic acid (**VI**) was prepared by a published procedure, purified by preparative HPLC (C_{18} column; CH_3CN/H_2O, 58:42), and recrystallized from hexane/benzene (1:1) (mp: 126–126.5°C; lit. (4), 125–126.5°C). The sodium salt of **VI** was prepared by titration of the acid with one equivalent of 1 M NaOH, followed by dilution with H_2O to give a 50 mM solution (<1% contamination by *p*-chlorocinnamate was detected by analytical HPLC).

Carboxypeptidase A (Cox preparation) was purchased from Sigma (lot 78 C-8055) as a crystalline suspension in toluene/water. Dialysis of 200 μl of the commercial suspension at 4°C against 1 M KCl/0.05 M Tris·HCl, pH 7.5 followed by centrifugation and transfer of the supernatant yielded the enzyme stock solution. The concentration (≈0.1 mM) of the enzyme was determined spectrometrically and the activity was determined by a standard assay procedure (18).

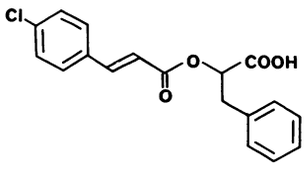
Benzoylglycyl-L-phenylalanine (**IV**) was purchased from Sigma and recrystallized from EtOAc [mp: 145–146°C; lit. (19), 146–147°C]. HPLC showed no detectible contamination by benzoylglycine. In calibration studies with authentic mixtures, as little as 0.1% benzoylglycine was easily detected.

Benzoylglycyl-β-L-phenyllactic acid (**V**) was purchased from Vega as the sodium salt. Conversion to the free acid was effected by careful addition of 1 M HCl to an aqueous solution



IV X = NH

V X = O



VI

of the salt followed by extraction with ether. The microcrystalline powder was dried *in vacuo* over P_2O_5. HPLC showed 0.3% contamination by benzoylglycine. This contamination was considered in calculating the isotope-partitioning effect.

MeOH–H_2O Competition in the Model System. An 8.7 mM solution of the model anhydride (**I**) in dimethyl sulfoxide (30 μl) was added to 1.0 ml of H_2O with zinc buffered at pH 6.3 [10 mM morpholinoethyl sulfate/5 mM LiOH/190 mM LiCl/10 mM $Zn(OAc)_2$] at 25.0°C. The anhydride hydrolyzed almost instantly, as indicated by the fading of the yellow color. Then, 2 ml of MeOH containing the same zinc/buffer as above was added. The UV/visible spectrum (from 280 nm to 450 nm) showed a shoulder at 290 nm ($\varepsilon = 1.6 \times 10^3$). When the solution of **I** was added to 2 ml of MeOH/zinc/buffer and this solution was then added to 1 ml of H_2O buffer, the UV/visible spectrum showed a peak with λ_{max} 341 nm ($\varepsilon = 2.4 \times 10^3$), as the result of methanolysis of the anhydride.

In a typical competitive hydrolysis/methanolysis experiment, the anhydride solution was added to a mixture of 2 ml of MeOH/zinc/buffer and 1 ml of H_2O/zinc/buffer. The ratio of rate constants for methanolysis to hydrolysis (15 ± 2) was determined from the product ratio obtained by measuring the absorption at 341 nm. In a similar experiment, a methanolysis/hydrolysis rate constant ratio of 15 was obtained from reaction of **I** in 1 ml of MeOH/zinc/buffer and 9 ml of H_2O/zinc/buffer. Addition of 80 μl of 0.5 M Na_4EDTA to the hydrolysis product of **I** resulted in a UV/visible spectrum change from a shoulder at 290 nm to a peak at 328 nm. Addition of EDTA to the methanolysis product of **I** resulted in a UV/visible spectrum change from a peak at 341 nm to a peak at 343 nm.

MeOH–H_2O Competition in the Carboxypeptidase A System. A 20-μl aliquot of a 50 mM aqueous solution of the sodium salt of the Makinen–Kaiser ester (**VI**) was added to a mixture of 50 μl of buffer (0.1 M Tris·HCl, pH 7.5/0.5 M NaCl), 20 μl of absolute MeOH, and 10 μl of enzyme stock solution (90 μM). The resulting mixture was incubated at 25°C for 2–2.5 hr, and then the product composition was analyzed by reversed-phase HPLC. Under these conditions, <0.14% methyl *p*-chlorocinnamate formed, which corresponds to $k_{MeOH}/k_{H_2O} \leq 0.009$. Authentic methyl ester, added to the reaction mixture, was not appreciably destroyed under these conditions.

Isotope-Partitioning Experiment. Typically, a $H_2{}^{16}O$–$H_2{}^{18}O$ reaction solution was prepared by mixing 200 μl of $H_2{}^{18}O$, 200 μl of 100 mM Tris buffer (0.1 M Tris·HCl, pH 7.5/1 M KCl), and 1 μl of the enzyme stock solution. To 50 μl of this mixture, substrate **IV** or **V** was added as either a solid or a solution (2 μl of 100 mM solution in dry dimethylformamide). After 5 min at room temperature, the reaction solution was frozen in a dry ice/acetone bath and then lyophilized overnight. The lyophilized residue was suspended in 100 μl of dry dimethylformamide, and diazomethane in argon was bubbled through the solution until the yellow color persisted. After 10–15 min, solvent and excess diazomethane were removed *in vacuo*. HPLC analysis of the reaction mixture showed >95% methylation of the benzoylglycine product. The residue was taken up in 250 μl of spectral grade CH_2Cl_2 and filtered.

The resulting solution (5 μl) was applied via a syringe to the glass rod of a Ross injector, the solvent was evaporated in a flow of helium, and the dried sample was then injected directly onto the gas chromatography column [10% SE-30 on 100–120 gaschrom Q; 6 ft (1.86 m) × 2 mm (i.d.) glass column]. The column effluent was monitored with a Ribermag 10–10 quadrupole mass spectrometer equipped with a PDP-8 Techtronix data processor. To avoid fragmentation, samples were run in the chemical ionization-negative ion mode with NH_3/He [0.2 atm (1 atm = 6.9 kPa)] as the reagent gas. Typically, a 5-μl injection of the sample entered the source of the mass spectrometer over a period of 1–2 min, during which time 200–500 measurements of P, P+2, and P+4 peaks (corresponding to *m/e* 192, 194, and 196 for methyl benzoylglycinate) were made. The ratios P+2/P and P+4/P were calculated from each measurement and were analyzed in sets of 20, each set with its own mean and SD. The mean for each set was independent of the amount of sample in the source. Weighted averaging (20) of these results yielded the isotope ratios, which were subsequently corrected for contributions from natural abundance ^{13}C and ^{2}H. The final isotope-partitioning values, based on the weighted average of the re-

sults from four to six injections, were calculated from

$$\frac{k_{16}}{k_{18}} = \frac{P}{P+2} \cdot \frac{H_2{}^{18}O}{H_2{}^{16}O}.$$

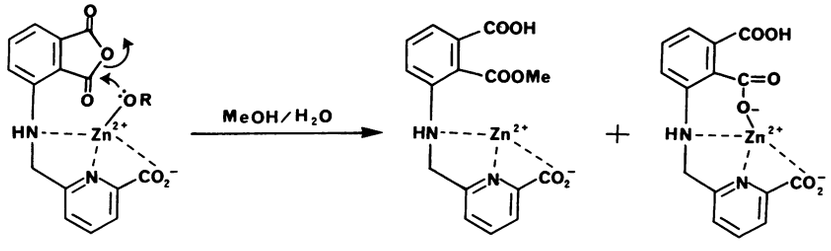
Equilibrium Isotope Effect. Reaction solution $H_2{}^{16}O/H_2{}^{18}O$ was prepared by mixing 100 μl of $H_2{}^{18}O$, 60 μl of $H_2{}^{16}O$, 200 μl of 100 mM Tris buffer, and 40 μl of the enzyme stock solution. To this mixture was added 20 μl of a 100 mM solution of **IV** in dry dimethylformamide. Fifty-microliter aliquots were taken at various times up to 1 day and frozen in a dry ice/acetone bath. The product was analyzed by gas chromatography/mass spectrometry as described above. Data for attainment of equilibrium of the exchange of the carboxyl oxygens of benzoylglycine in $H_2{}^{16}O/H_2{}^{18}O$ can be fit according to an integrated first-order rate equation (see Fig. 1). The half-time for the attainment of equilibrium is 5 hr. The equilibrium isotope effect for this process, based on the ^{18}O enrichment extrapolated to infinite reaction time, is 0.98 ± 0.01.

Determination of $H_2{}^{16}O$–$H_2{}^{18}O$ by the CO_2 Method. To 100 μl of the reaction mixture for the isotope-partitioning experiment (without substrate), ≈0.1 mg of $NaHCO_3$ was added. This sample was sealed, let stand overnight, then attached to the Ribermag reference line (FC43), and placed under 1 atm of helium. After 30 min, the valve to the source of the mass spectrometer was opened and peaks corresponding to *m/e* 44, 46, and 48 were analyzed (positive-ion chemical ionization). $H_2{}^{16}O/H_2{}^{18}O$ was calculated from this analysis, using the fractionation factor for CO_2 exchange of 1.041 (21, 22):

$$\frac{H_2{}^{16}O}{H_2{}^{18}O} = \frac{1.041(P+2)}{2(P+4)}.$$

RESULTS AND DISCUSSION

MeOH–H_2O Partitioning in a Model System and in the Enzyme. If an anhydride intermediate is formed when carboxypeptidase A hydrolyzes peptides and related esters, that intermediate itself must be hydrolyzed with the Zn^{2+} ion as the principal catalyst. The glutamate is presumably part of the anhydride, while the tyrosine is not required for ester substrates so it cannot be invoked in the cleavage of a common intermediate. Thus, the model system (**I**) described some years ago (23), in which zinc catalyzes the cleavage of a bound anhydride substrate, is relevant to the proposed mechanism. We had evidence that the mechanism used by zinc to cleave this anhydride was the delivery of a zinc-bound hydroxide group to the adjacent anhydride carbonyl; this is now confirmed by our studies on zinc-catalyzed methanolysis of this substrate.

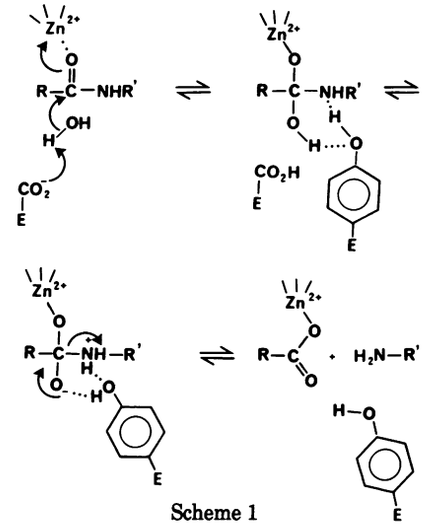


I·Zn^{2+} II·Zn^{2+} III·Zn^{2+}

We find that zinc can catalyze the delivery of MeOH to the substrate to form a monomethyl ester (**II**) whose UV spectrum (λ_{max} 343 nm) is consistent with the structure. For comparison, methyl *o*-aminobenzoate has λ_{max} 336 nm but methyl *m*-aminobenzoate has λ_{max} 318 nm, while the anion of the hydrolysis product (**III**) has λ_{max} 328 nm, *o*-aminobenzoate anion has λ_{max} 324, and *m*-aminobenzoate anion has λ_{max} 304 nm. As described above, the UV change when Zn^{2+} is removed from **II** with EDTA is negligible, as expected with a weakly bound ester group while, for the hydrolysis product **III**, removal of Zn^{2+} from the bound COO^- ion leads to a substantial UV shift.

In a competition experiment, the zinc-catalyzed reaction with the anhydride group shows a significant preference for MeOH. In aqueous MeOH, the rate constant for methanolysis is larger than that for hydrolysis of **I** by a factor of *ca.* 15:1. If reactions of carboxypeptidase involve zinc-catalyzed cleavage of an anhydride intermediate, one might expect a similar preference for MeOH unless special factors intervene.

As previously reported (6), MeOH is not competitive with H_2O toward either normal peptides or related esters, and we have now examined ester **VI** for which an anhydride intermediate has been proposed. We find in a MeOH–H_2O competition experiment with this substrate that there is no detectable enzyme-catalyzed formation of a methyl ester. There is a small amount of spontaneous uncatalyzed methanolysis of the substrate, but the enzyme-catalyzed term shows at least a 100-fold preference for H_2O over MeOH. The most obvious conclusion from these studies is that none of the substrates for carboxypeptidase are hydrolyzed through anhydride intermediates.



Scheme 1

The preference for H_2O in carboxypeptidase A-catalyzed cleavages can be explained by a general base mechanism (Scheme 1) in which both protons of the H_2O are required (6). In a tetrahedral intermediate formed by the general base-catalyzed delivery of a water molecule, reversal of that intermediate to the starting material is expected unless the second proton can transfer so that the product would be directly a COO^- *anion* bound to the zinc atom. No such possibility exists with MeOH. According to this mechanism, attack of MeOH would form a tetra-

hedral intermediate that would simply reverse, leading to no productive reaction.

It is not surprising that, in the model system in which zinc catalyzes the cleavage of an anhydride (I), there is no such preference for H_2O even though here also a second proton transfer could have produced a product (III) with a COO^- anion coordinated to the zinc, while MeOH attack forms (II), a poorly binding ester group. Once a MeO^- ion has added to an anhydride carbonyl to form a tetrahedral intermediate, it would not be expected to reverse. The intermediate would certainly decompose in the forward direction with loss of the excellent COO^- leaving group and concomitant formation of the methyl ester. Thus, with a highly reactive intermediate such as an anhydride, it is expected that MeOH should be able to compete with H_2O because attack on the intermediate is rate determining and irreversible, so the second proton transfer cannot be required for overall forward reaction.

The finding that no substrate cleaves enzymatically with detectable participation by MeOH, though it argues against an anhydride intermediate for any of them, did not permit us to use MeOH–H_2O competition to test for a common intermediate. For this reason, we turned to a competition guaranteed to work, that between $H_2{}^{16}O$ and $H_2{}^{18}O$.

$H_2{}^{16}O$–$H_2{}^{18}O$ Partitioning. To our knowledge, no one has yet reported the study of isotope effects in the enzyme-catalyzed hydrolysis of substrates by using water containing $^{16}O/^{18}O$ mixtures. For that matter, in spite of the potential that such a tool might have for elucidating mechanisms, no such studies seem to have been performed for nonenzymatic hydrolysis reactions either. It is easy to understand this. In general, ^{18}O kinetic isotope effects are small, <10% (24), and the experimental difficulties are considerable. Chief among them is the requirement to control contamination by adventitious water. In addition, there is a requirement that the product of the hydrolysis be analyzed for isotope composition with high precision to establish the small isotope effects involved reliably. We have now developed this tool for the study of carboxypeptidase A; the technique may be generally useful in the study of enzymatic and nonenzymatic hydrolysis reaction mechanisms.

Benzoylglycyl-L-phenylalanine (IV) and benzoylglycyl-β-L-phenyllactate (V) were hydrolyzed by carboxypeptidase in H_2O containing a known amount of ^{18}O, and the resulting hydrolysis products were directly converted to the corresponding methyl esters with diazomethane. We were able to determine the ^{18}O content of the benzoylglycine methyl ester derived from each of these substrates with high precision on a quadrupole mass spectrometer adapted so that we could do repeated injections, multiple analyses within a single injection, and computer averaging of the results, in a fashion similar to that reported by Kwart (25) and others (26, 27). With care, we find that we can determine the ^{18}O content of such samples with 0.2% reproducibility. The ^{18}O content of the H_2O used in the reaction was determined directly on the reaction mixture by equilibration of the system with CO_2 (21, 22). The CO_2 ^{18}O content was also determined in the quadrupole mass spectrometer and used with the known equilibrium isotope effect (21, 22) for the exchange between H_2O and CO_2 to calculate the composition of the H_2O, which was set near 50% for the kinetic runs.

Hydrolysis of the amide substrate (IV) showed a kinetic isotope effect of 1.019 ± 0.002, by which ^{16}O was preferentially incorporated into product relative to its composition in the starting H_2O. These numbers were taken after 50–70% hydrolysis of the substrate. Because of the reversibility of amide hydrolysis reactions (6), it was important not to allow the hydrolysis products to equilibrate by standing longer. On equilibration, the amide showed an isotope effect of 0.98 ± 0.01. The inverse effect is expected for a H_2O–COO^- equilibration. In contrast to the amide, the ester substrate (V) showed a kinetic isotope effect of 0.998 ± 0.002—i.e., no effect within experimental error. The difference between the 2% effect with an amide and the noneffect with the ester is 10 SD units.

Although the reproducibility of the data shows that random errors are small compared with the observed 2% isotope effect for the amide substrate, one must consider possible systematic errors. A 1% contamination of the $H_2{}^{18}O$ by $H_2{}^{16}O$ would lead to an erroneous 2% isotope effect, so that $H_2{}^{18}O/H_2{}^{16}O$ ratio was directly measured and not simply calculated. Contamination of the starting substrates by already hydrolyzed products could introduce an error approximately twice the magnitude of the contamination. The HPLC analysis showed negligible contamination of the amide but 0.3% contamination of the ester. Thus its observed isotope effect of 0.998 must be corrected to 0.992. The amounts of dried dimethylformamide or $NaHCO_3$ introduced were so small that no significant contamination by $H_2{}^{16}O$ could have occurred. Furthermore, the finding of an expected (21) *inverse* isotope effect of sensible magnitude when amide hydrolysis was allowed to come to equilibrium excludes contamination that would contribute false positive isotope effects. As Fig. 1 shows, the kinetic points at various times show an excellent fit to the expected first-order approach to equilibrium, suggesting the high reliability of these data.

These findings rule out many mechanistic possibilities. For instance, it has been proposed that amides and esters both hydrolyze through a common anhydride intermediate (28), possibly with different rate-determining steps (29). Such a common intermediate is ruled out by the finding of different kinetic isotope fractionations unless the alcohol or amine leaving groups are still bound in the pocket of the enzyme and in some way affect the isotopic preferences for the anhydride hydrolysis step. Furthermore, the previous studies (6) on ^{18}O exchange reactions excluded an anhydride intermediate for this enzyme unless the H_2O molecule involved in cleaving that anhydride is trapped and not in free exchange with other H_2O molecules. The kinetic isotope effect of 1.02 shows that when the amide substrate (IV) reacts with H_2O there is a selection among several H_2O molecules, so that an isotope effect can be manifest, rather than automatic cleavage with whichever H_2O molecule happens to be trapped. Of course, since the nature of the putative trapping is not easily specified, it is possible that there could be a 2% equilibrium isotope effect involved in such trapping. However, this should also then be seen with the cleavage of the ester substrate (V), but no such isotope effect was observed. Thus in their simplest interpretation the isotope effects we have found here close the trapped-H_2O loophole, which was

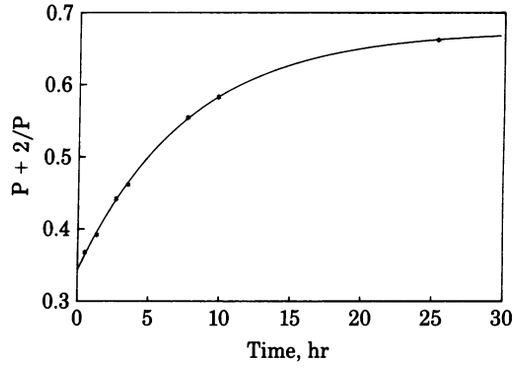


FIG. 1. The carboxypeptidase-catalyzed further incorporation of ^{18}O into benzoylglycine after initial rapid hydrolysis of benzoylglycylphenylalanine in $H_2{}^{16}O/H_2{}^{18}O$. The points are the experimental ratios of singly labeled to unlabeled benzoylglycine; the solid line is the best fit calculated for a first-order rate process. The excellent agreement suggests that the experimental method is reliable.

the principal problem with the previous evidence against an anhydride intermediate.

The indirect nature of most evidence about reaction mechanisms means that there is another possibility, however. If the *hydrolysis* of an anhydride intermediate were *rate limiting* with amide substrates, then it would be possible that such cleavage could involve a trapped H_2O molecule, trapped for instance on the zinc. In the presence of the bound amide, the anhydride would reverse to bound amide substrate, permitting exchange of this trapped H_2O molecule and allowing a kinetic isotope effect to be established. Thus, although our evidence strongly argues against an anhydride intermediate in the action of carboxypeptidase A, such an intermediate would still be possible if it were formed in a rapid reversible step from the bound amide, with the hydrolysis of this anhydride being rate limiting. However, in view of all the evidence against such an anhydride intermediate, it does seem incumbent on the proponents of this mechanism to furnish some direct evidence for its formation in the cleavage of normal (or abnormal) substrates.

Other Arguments for the General Base Mechanism. The x-ray studies of thermolysin by Kester and Matthews (30) have been interpreted by them as favoring the general base mechanism for this enzyme, which seems closely related to carboxypeptidase. In addition, a transition-state analog constructed by Jacobson and Bartlett (31) was based on the general base mechanism for carboxypeptidase, and its effectiveness and pH-binding profile make most sense in terms of a resemblance to the tetrahedral intermediate formed by delivery of a H_2O molecule to the scissile carbonyl group in the enzyme mechanism of Scheme 1. Another argument for the general base mechanism derives from thermodynamic considerations. The leaving group of the substrate has two specific interactions (hydrophobic pocket, ionic bond) with the pocket of the enzyme and might remain bound even after an acyl–enzyme intermediate were formed. Indeed such binding can be invoked to explain why release of the leaving group has not yet been detected when the Makinen–Kaiser ester purportedly forms its intermediate; it also must be invoked in the only loophole in our current work that excludes trapped H_2O because of the observed kinetic isotope effects. However, if the leaving group is not released, the principal driving force for the formation of a high-enthalpy anhydride intermediate from a relatively stable peptide or ester substrate is no longer present.

The entropy advantage of the two-step mechanism involves the loss of part of the substrate when the intermediate is formed and, if that piece is still bound to the enzyme, one would not expect a pathway involving a high-energy intermediate to have any catalytic advantage. As was pointed out earlier, this is the general observation with simple model systems: fragmentations can lead to high-energy intermediates, but paths are not preferred in which the leaving group is still bonded in an intermediate of much higher enthalpy than the substrate. Thus all of the available evidence best fits a general base mechanism, as in Scheme 1.

CONCLUSIONS

(*i*) The finding that in a model system the zinc-catalyzed cleavage of an anhydride shows a *preference* for MeOH over H_2O, while no substrate for carboxypeptidase A can utilize MeOH detectably in competition with H_2O, casts doubts on the use of an anhydride intermediate in the mechanism of cleavage of any substrate for the enzyme.

(*ii*) The kinetic isotope effect involved in partitioning $H_2{}^{16}O$ and $H_2{}^{18}O$ can be used as a tool for investigating hydrolysis mechanisms.

(*iii*) The finding of different isotope effects in the cleavage of normal amide and ester substrates rules out previously invoked mechanisms in which they proceed through a common intermediate. The data also close the "trapped-H_2O loophole" in previous studies addressing the question of an intermediate in these processes. These isotope effects, and the previous studies on oxygen exchange reactions, exclude a common anhydride intermediate except for the one specific possibility that this anhydride intermediate is reversibly formed with amide substrates and undergoes rate-limiting hydrolysis.

(*iv*) The most reasonable mechanism consistent with all the data is the general base process of Scheme 1, which does not involve an acyl–enzyme intermediate. This is also consistent with work with transition-state analogs and with other zinc enzymes and with thermodynamic arguments based on the structure of carboxypeptidase itself.

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