Table I: Kinetic Data on the Cleavage of the Maleamic Acids, 25.0 °C, 1 M H_2O in CH_3CN

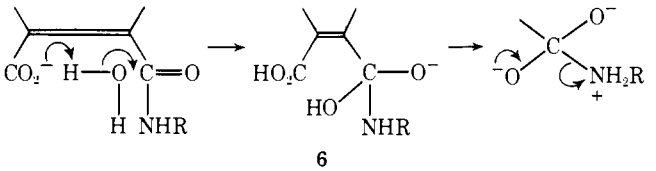
Compd	Mp, °C	10^4k (s^{-1}) with HOAc–KOAc Buffer[a] 10:1	1:10	1:50
1[b]	151–152	75.3	~0.1	0.0314
2[b]	118–120	45.5	1.80	2.07[d]
3[c]	129–136	41.6	5.12	4.55[e]
4[b]	123–125	28.8	~0.07	0.0238
5[c]	107–110	20.1	0.866	0.959

[a] Ratio 10:1 indicates the buffer ratio of HOAc/KOAc at a buffer concentration of 5×10^{-3} M HOAc, 5×10^{-4} M KOAc with 1×10^{-4} M substrate. 1:50 is 5×10^{-3} M KOAc, with 1×10^{-4} M substrate whose ammonium cation generates the HOAc. Rate constants are the average from at least three runs (except for the two approximate values for 1 and **4**) and had standard deviations of 6–16%. [b] Characterized by NMR spectra, and C, H, N analyses within 0.3% of theoretical. [c] Characterized by NMR spectra, correct H, N (and Br) analyses, but low C analyses suggesting some hydration or other impurity. [d] k_{H_2O}/k_{D_2O} is 1.47. [e] k_{H_2O}/k_{D_2O} is 2.27.

maleic anhydride, and again no catalytic effect was observed from the phenolic hydroxyls of compounds **2, 3,** or **5**.

However, the behavior of these compounds diverged in 1:10 HOAc/KOAc or 1:50 HOAc/KOAc. Compounds **1** and **4**, without the phenolic hydroxyl, now underwent very slow hydrolysis, in parallel with the behavior of the free carboxylate ions in aqueous solution. In these (increasingly) more basic media, protonation of the leaving amino group is now a problem. By contrast, the phenolic compounds **2, 3,** and **5** underwent a moderate decrease in rate in the 1:10 medium and no further decrease in the 1:50 medium. The phenolic hydroxyls can now supply the required acid proton, and acid from the medium is no longer required.[8] As the data in Table I show, the catalytic effects of these hydroxyl groups are substantial. Comparing **2** with **1** in the neutral (1:50) solution, the acceleration is 66-fold. Thus, if carboxypeptidase actually cleaves esters and peptides by similar mechanisms, except that peptide hydrolysis is also assisted by a tyrosine hydroxyl, that assistance could bring the peptide rates up to those of esters.[9]

An additional striking change on converting the medium from the 10:1 acidic buffer to the neutral 1:10 or 1:50 buffer is that the substrate abandons the anhydride mechanism. Anhydride cannot be detected as a reaction intermediate with **2, 3,** or **5** by spectroscopy or by trapping with added simple amines, although authentic dimethylmaleic anhydride can be detected in both these ways if it is added to the medium. We conclude that in this model system, the nucleophilic catalytic role played by the carboxylate ion at low pH is supplanted by another catalytic role, presumably general base delivery of water,[8] in the pH region corresponding to neutrality. Such a change in mechanism at higher pH is known for other neighboring group catalysts,[10] and can be understood in terms of the energetics of the individual steps. In essence, a leaving group protonated by the weak phenolic hydroxyl cannot be ejected to form the high energy anhydride, only to form the more stable carboxylate ion. This implies that in the tetrahedral intermediate **6** a proton must next be removed from the hydroxyl, as well as added to the nitrogen. The phenolic group could assist in both of these processes.[11]



6

Thus, these model systems utilize two of the three known functional groups of carboxypeptidase A to catalyze an amide hydrolysis. Furthermore, they use either of the two mechanisms generally considered for the enzyme, depending on the reaction conditions. This again calls attention to the necessity to resolve the mechanistic ambiguities with the enzyme itself. The accompanying communication[12] indicates that the enzyme, at neutrality, apparently parallels our model system in utilizing nonnucleophilic catalysis by carboxylate.

Acknowledgment. Financial assistance of this work by the National Institutes of Health is gratefully acknowledged.

References and Notes

(1) For reviews, see (a) E. T. Kaiser and B. L. Kaiser, *Acc. Chem. Res.*, **5**, 219 (1972); (b) W. N. Lipscomb, *Tetrahedron*, **30**, 1725 (1974); F. A. Quiocho and W. N. Lipscomb, *Adv. Protein Chem.*, **25**, 1 (1971).
(2) Reference 1b and work cited therein. However, see also ref. 4a.
(3) B. L. Vallee, J. F. Riordan, and J. E. Coltman, *Proc. Nat. Acad. Sci. U.S.A.*, **49**, 109 (1963).
(4) (a) J. T. Johnson and B. L. Vallee, *Biochemistry*, **14**, 649 (1975); (b) E. T. Kaiser, private communication. (c) Note that in our proposals in this and the accompanying communication the phenolic catalysis of proton transfer should be a subsequent fast step, not the rate-determining step. Therefore conditions which simply slow that fast step, e.g., partial titration of the phenol, will not necessarily slow the overall rate. Of course blocking phenol catalysis entirely, by acetylation of the phenolic hydroxyl, could finally make the proton transfer step rate determining.
(5) For the previous publication, see R. Breslow, D. E. McClure, R. S. Brown, and J. Eisenach, *J. Am. Chem. Soc.*, **97**, 194 (1975).
(6) A. J. Kirby and A. R. Fersht, *Prog. Bioorg. Chem.*, **1**, 28 (1971). Two recent studies of bifunctional catalysis of maleamic acid hydrolysis have been reported during the course of our work: (a) M. F. Aldersley, A. J. Kirby, P. W. Lancaster, R. S. McDonald, and C. R. Smith, *J. Chem. Soc., Perkin Trans. 2*, 1487 (1974); (b) A. J. Kirby, R. S. McDonald, and C. R. Smith, *ibid.*, 1495 (1974). In this work the nucleophilic (anhydride) mechanism was maintained, at low pH, but proton transfers were catalyzed by an additional catalytic group.
(7) Although "pH" can be read on a meter in such media, it seems best to consider the state of ionization of the catalytic groups. This should parallel the state of ionization of the buffer acid.
(8) Two control reactions help establish this mechanism. *N*-Benzoyl-*o*-hydroxybenzylamine is completely stable over many days in our medium; thus the carboxylate in **2** plays a role. Furthermore, reaction of dimethylmaleic anhydride with phenoxide ion produces the unstable phenyl monoester of dimethylmaleic acid; in the 1:50 HOAc/KOAc medium this rapidly re-forms the anhydride. Since hydrolysis of amide **2** does not proceed through the anhydride under these conditions, it must not be using the phenolic group as a nucleophile to form an intermediate phenyl ester.
(9) Approximately 10^3 would be needed if peptide substrates are to be brought to the reactivity of ester substrates.
(10) E.g., A. R. Fersht and A. J. Kirby, *J. Am. Chem. Soc.*, **90**, 5826 (1968), describe the change from nucleophilic catalysis in aspirin acid to general base catalysis in its anion.
(11) The somewhat greater reactivity of **3**, the bromophenol, than of **2** indicates some net proton transfer from the phenolic group. Thus proton transfer to N is more advanced than proton recovery from the carbinol group in the tetrahedral intermediate.
(12) R. Breslow and D. Wernick, *J. Am. Chem. Soc.*, following paper in this issue.

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On the Mechanism of Catalysis by Carboxypeptidase A

Sir:

Two general mechanisms have been proposed[1] for hydrolytic reactions catalyzed by bovine pancreatic carboxypeptidase A (CPA, E.C.3.4.12.2). In one the γ-carboxylate of Glu-270 acts as a nucleophile at the scissile carbonyl, forming an anhydride intermediate;[2] in the other Glu-270 acts as a general base, delivering nucleophilic water instead. As the accompanying communication indicates,[3] we have model systems for both of these mechanisms. Work on the enzyme now allows us to choose between them.

Table I. Turnover Numbers for CPA Catalyzed ^{18}O Exchange[a]

N-Benzoylglycine (mM)[b]	Added component (mM)	*V/E* (min^{-1})
7.6	None	<0.01
10	L-Phenylalanine (10)	8
10	L-Phenylalanine (1)	2
4.1	L-Phenylalanine (1)	0.8
7.6	L-β-Phenyllactic acid (10)	≤0.02
7.1	L-β-Phenyllactic acid (10) + L-phenylalanine (5)	0.03
10	*p*-Hydroxyphenylpropionic acid (10)	<0.2
8.3	L-Leucine (10)	0.6
7.6	L-Proline (10)	<0.01
7.6	Glycine (10)	<0.02
7.6	L-Alanine (10)	<0.06
7.6	L-Threonine (11)	<0.09
8.4	*N*-Methyl-L-leucine (8)	0.02
N-Acetylglycine (mM)[c]		
10	None	<0.008
10	L-Phenylalanine (10)	0.6
10	L-β-Phenyllactic acid (10)	<0.004
10	L-Leucine	0.02

[a] At 20.0°, pH 7.50, μ = 0.1, *N*-ethylmorpholine–acetic acid buffer, [CPA] = 1×10^{-6} to 3×10^{-5} M. *V/E* is the bulk turnover number. [b] Initially with 75.5% enrichment of one ^{18}O in the carboxyl, by hydrolysis of the methyl ester with $Na^{18}OH$. Assay of the reactions by freeze-drying and CI MS of the residue. [c] Initially with 15.9% enrichment of one ^{18}O in the carboxyl. Assay of the reactions by freeze-drying, methylation with CH_2N_2, and analysis by GC/CI MS.

Table II. Methanol–Water Competition with Some Hydrolytic Enzymes

Enzyme	k_{MeOH}/k_{H_2O}
α-Chymotrypsin	39–584[a]
Trypsin	82[b]
CPA-esterase	<0.06[c]
CPA-peptidase	<0.0003[d]

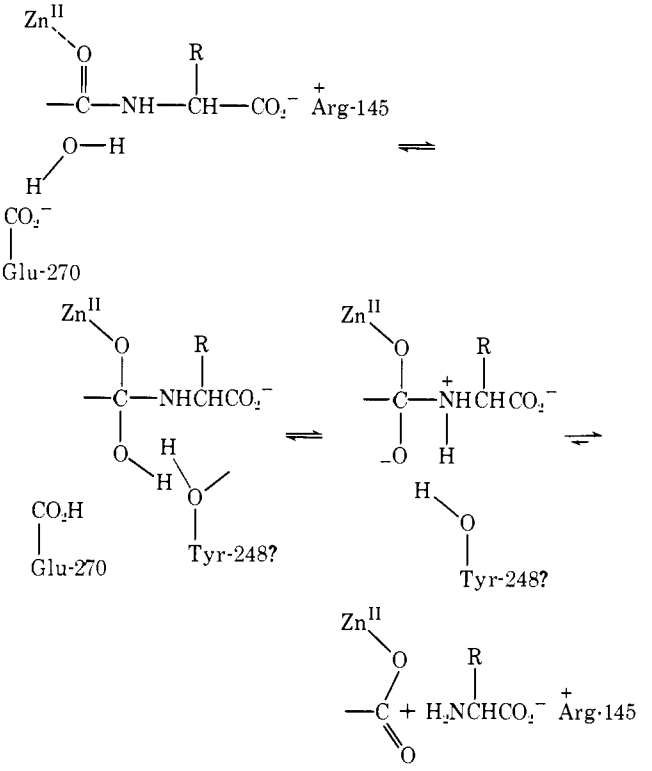
[a] For several substrates: M. L. Bender, G. E. Clement, C. R. Gunter, and F. J. Kezdy, *J. Am. Chem. Soc.*, 86, 3697 (1964). [b] M. L. Bender, "Mechanisms of Homogeneous Catalysis from Protons to Proteins", Wiley, New York, N.Y., 1971, p 516. [c] pH 7.5, 25°. [d] pH 7.5, 20°.

The only direct evidence in favor of an anhydride (acyl-enzyme) intermediate is the report by Ginodman et al.[4] that CPA catalyzes ^{18}O incorporation from $H_2{}^{18}O$ into the carboxyl of *N*-acyl amino acids, such as *N*-acetylphenylalanine. Considering the acyl enzyme mechanism (eq 1), it is obvious that the enzyme could incorporate ^{18}O into $AcPheCO_2H$ by reversible resynthesis of the intermediate. However, if an acyl-enzyme intermediate is not involved, a compound such as $AcPheCO_2H$ could still undergo ^{18}O exchange *if another amino acid were present*, by enzyme-catalyzed reversible synthesis of a peptide. We find that this is actually the case, and that the previously reported[4] ^{18}O exchange must have involved additional amino acid impurities[5] to permit CPA catalyzed peptide synthesis.

$$\text{AcPheCO-NHCHCO}_2\text{H} \xrightarrow{\text{CPA}} \text{NH}_2\text{CHCO}_2\text{H} + \text{AcPheCO-CPA} \overset{H_2{}^{18}O}{\rightleftharpoons} \text{AcPheCO}_2\text{H} + \text{CPA} \quad (1)$$

Our clearest results are seen with *N*-benzoylglycine,[6] which we have synthesized with 76% ^{18}O in the terminal carboxyl by $H_2{}^{18}O$ hydrolysis of the methyl ester. As Table I shows, CPA catalyzes the exchange of ^{18}O from this labeled *N*-benzoylglycine but only in the presence of added phenylalanine or leucine. In their absence, no ^{18}O exchange can be detected. With 3-phenyllactic acid instead, ^{18}O exchange is too slow to be detected and must be at least 400 times slower than that in the presence of phenylalanine. Similar results were obtained with ^{18}O labeled *N*-acetylglycine (Table I). All this is as expected if the mechanism of ^{18}O exchange involves resynthesis of a substrate. Since the enzyme catalyzes the hydrolysis of esters and amides with approximately the same rate, while the equilibrium constant differs by a factor of ca. 10^3, we would expect the enzyme catalyzed resynthesis of an ester substrate such as *N*-benzoylglycyl phenyllactate to be approximately 1000 times slower than that of a peptide substrate such as *N*-(*N*-benzoylglycyl)phenylalanine. On the other hand, if an acyl-enzyme intermediate were involved, as in eq 1, we would expect that ^{18}O exchange would occur in the absence of either phenylalanine or phenyllactic acid. Thus, an acyl-enzyme intermediate is very unlikely[7] in the hydrolysis reactions catalyzed by CPA.

Scheme I



Further information about the mechanism can be obtained by examining the ability of methanol to substitute for water. Using L-*O*-acetylmandelate tritiated in the acetyl group we have examined the competition between methanolysis and hydrolysis catalyzed by CPA in 5 M aqueous methanol. No labeled methyl acetate was produced above the amount formed by a small amount of spontaneous methanolysis of the substrate, so that k_{CH_3OH}/k_{H_2O} is less than 0.06. This can be contrasted (Table II) with the efficient competition of methanol with water in many solvolyses catalyzed by other proteinases. Methanolysis of peptide substrates of CPA cannot be directly observed because the equilibrium constant is unfavorable, but again an enzyme must be able to catalyze a reaction in either direction. Thus, we examined the ability of CPA to synthesize *N*-benzoylglycylphenylalanine from *N*-benzoylglycine methyl ester and phenylalanine, then hydrolyze the peptide, leading to overall hydrolysis of the methyl ester. This could most conveniently be performed by simultaneously monitoring the phenylalanine-promoted ^{18}O exchange from labeled *N*-ben-

zoylglycine as described above.[8] No detectable hydrolysis of the methyl ester occurred with CPA and phenylalanine. By comparison with the ^{18}O exchange rate, it could thus be determined that k_{CH_3OH}/k_{H_2O} is less than 0.0003.

Thus, the enzyme cannot incorporate methanol in the transition state of the reaction (run in either direction) for either ester or peptide substrates.[9] This suggests that removal of both protons of water is required in the transition state for hydrolysis.

From these data all together, we suggest the mechanism shown in Scheme I for the hydrolysis of peptide substrates. The glutamate carboxylate acts as a general base to deliver nucleophilic water to the carbonyl, but if this were instead methanol then the first step would simply reverse. Only a second deprotonation could drive the reaction in the forward direction, and this proton transfer might well involve the tyrosine hydroxyl[10] as a bridge between the OH and the N as we have suggested for our model system.[3]

Much controversy[11] has surrounded the question of whether Arg-145 or Zn^{2+} is the binding site for substrate carboxylate. Our mechanism indicates that *both* are true, the Zn^{2+} binding the carboxylate of one hydrolysis product which is thus the substrate for the *reverse* reaction. In general one would expect that an exopeptidase should have two alternate binding sites, separated by a distance corresponding to one residue in the substrate, as in this mechanism.

Acknowledgment. Technical assistance by Vinka Parmakovich and financial support by the National Institutes of Health are gratefully acknowledged.

References and Notes

(1) For reviews, see (a) E. T. Kaiser and B. L. Kaiser, *Acc. Chem. Res.*, **5**, 219 (1972); (b) W. N. Lipscomb, *Tetrahedron*, **30**, 1725 (1974); (c) F. A. Quiocho and W. N. Lipscomb, *Adv. Protein Chem.*, **25**, 1 (1971).

(2) Our model studies (R. Breslow, D. E. McClure, R. S. Brown, and J. Eisenach, *J. Am. Chem. Soc.*, **97**, 194 (1975)) show that an anhydride intermediate cannot be excluded by the failure of trapping experiments.

(3) R. Breslow and D. E. McClure, *J. Am. Chem. Soc.*, preceding paper in this issue.

(4) L. M. Ginodman, N. I. Mal'tsev, and V. N. Orekhovich, *Biokhimiya*, **31**, 1073 (1966); *Biochemistry (USSR)*, **31**, 931 (1966). It is interesting that the Russian authors, despite their ^{18}O exchange results, concluded that an acyl–enzyme intermediate was unlikely on the basis of other evidence.

(5) *N*-Acetylphenylalanine is hydrolyzed by the enzyme rapidly enough (J. E. Snoke and H. Neurath, *J. Biol. Chem.*, **181**, 789 (1949)) to produce the phenylalanine needed to account for the reported ^{18}O exchange. Such partial hydrolysis can explain some of the data in ref 4, but not that for *N*-acetylglycine. Of course other amino acids are likely contaminants in peptidase preparations.

(6) *N*-Acylglycines are not hydrolyzed at an appreciable rate by carboxypeptidase A. As expected from this fact, assuming that it is resynthesis of peptides which accounts for the catalytic effect of added amino acids on ^{18}O exchange in *N*-acylamino acids, glycine is not an effective catalyst.

(7) It is actually excluded, except for the possibility that acyl–enzyme hydrolysis and resynthesis are strongly *catalyzed* by enzyme-bound amino acids, but not by enzyme-bound hydroxy acids. At this point Occam's razor must be applied. Note that the effectiveness of amino acids (Table I) correlates with their expected effectiveness in peptide resynthesis, as judged by enzymatic hydrolysis rates for the corresponding *N*-acyl amino acid linkages. This is particularly striking in the comparison of L-leucine (acyl leucines are good substrates) with *N*-methyl-L-leucine (the corresponding peptides are poor substrates).

(8) $[Bz\text{-}Gly\text{-}CO^{18}OH, 76\%\ ^{18}O] = 9.8 \times 10^{-3}$ M, $[Bz\text{-}Gly\text{-}CO_2CH_3] = 9.8 \times 10^{-4}$ M, $[Phe] = 9.8 \times 10^{-4}$ M, $[CPA] = 9.8 \times 10^{-7}$ M, pH 7.5, $\mu = 0.1$, *N*-ethylmorpholine–acetic acid buffer, 20.0°. Aliquots were freeze-dried and the residues chromatographed, then submitted to mass spectral and uv assay. Taking V_{hyd} as the observed initial rate of ester hydrolysis, k_0 as the first-order rate constant for ^{18}O exchange, and K_{hyd} as the equilibrium constant for hydrolysis of the ester (8×10^4 M at pH 7.5), the ratio of methanolysis and hydrolysis rate constants normalized to equal concentrations of methanol and water is

$$k_{MeOH}/k_{H_2O} = \frac{V_{hyd} \cdot 55\ M}{2k_0K_{hyd}[ester]_{initial}}$$

(9) Other workers have apparently[1c] failed to observe methanolysis of substrates; for peptides the reaction would be endothermic. We find that *no* nucleophiles (NH_3, NH_2OH, $NaBH_4$) except H_2O are used by CPA, making this aspect of its substrate requirement completely specific.

(10) Direct evidence for the involvement of Tyr-248 is not solid, although its acetylation leads to loss[1c] of peptidase activity by CPA.

(11) Cf. D. S. Auld and B. Holmquist, *Biochemistry*, **13**, 4355 (1974), and references therein.

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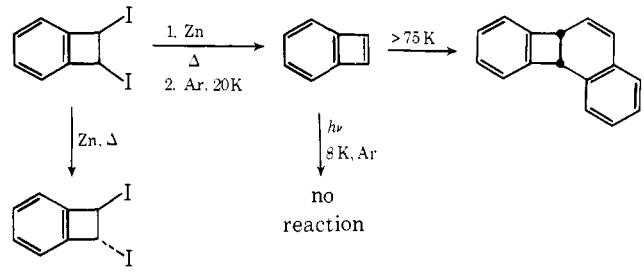
Benzocyclobutadiene

Sir:

We wish to report the direct spectroscopic observation of benzocyclobutadiene. Benzocyclobutadiene has been the subject of an extensive literature.[1] A variety of methods for generating benzocyclobutadiene as a reactive intermediate is known, but direct observation of this intermediate has not been possible.[1-5]

We have developed a method for generating clean samples of benzocyclobutadiene matrix isolated in argon. The apparatus is shown in Figure 1. *cis*-1,2-Diiodobenzocyclobutene is heated to 65 °C at 10^{-6} mm by the first heater to provide a sufficient rate of sublimation. The diiodide vapor is passed over zinc powder heated to 230 °C by the second heater. The vapor is then passed through a zone cooled by acetone evaporation. Small quantities of two products, benzocyclobutadiene dimer and *trans*-diiodide, deposit in the cooled region. The matrix deposited on the cesium iodide plate is free of both dimer and *trans*-diiodide. After deposition, the vacuum shroud is rotated 90° for infrared spectroscopic observation.[6] The same procedure is used for ultraviolet spectroscopic observation except that a sapphire plate is used.[6] The infrared spectrum of benzocyclobutadiene is shown in Figure 2 and the ultraviolet spectrum in Figure 3. The species in the matrix is identified as benzocyclobutadiene by the thermal dimerization above 75 K to the known benzocyclobutadiene dimer. The disappearance of the infrared and ultraviolet absorption bands due to benzocyclobutadiene and the concurrent appearance of the absorption bands due to the dimer establish the identity of the matrix isolated species as benzocyclobutadiene. In the infrared experiment, the dimer was washed from the window and further characterized by comparison of mass spectra, ultraviolet spectra, and thin layer chromatographic behavior in five solvent systems with authentic benzocyclobutadiene dimer. Attempts to degrade benzocyclobutadiene to benzyne and acetylene by ultraviolet irradiation were unsuccessful. Benzocyclobutadiene is quite stable to irradiation in an argon matrix.



The most intense band (737 cm^{-1}) in the infrared spectrum of benzocyclobutadiene is the deformation mode due to the four carbon–hydrogen bonds in a 1,2-disubstituted benzene. The band at 700 cm^{-1} is probably one of the deformation modes of the olefinic carbon–hydrogen bonds (cf. 650 and 570 cm^{-1} for the in-plane and out-of-plane modes for cyclobutadiene[7,8]). The ultraviolet spectrum of benzocyclobutadiene (λ_{max}^{Ar} 243, 246.5, 256, 264, 270, 281.5, and