Hans Brintzinger for disclosing experimental results prior to publication.

Supplementary Material Available: Five tables, listing interatomic distances and angles, fractional coordinates, and intensity data (22 pages). Ordering information is given on any current masthead page.

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(7) W.-S. Lee and H. Brintzinger, manuscript submitted for publication. We find the neutral complex **1** to be very air sensitive; in the absence of air, it decomposes at a moderate rate at high concentration in solution, but appears to be stable at room temperature in the solid state. It does not react with weak ligands such as CH_3CN or THF but is converted rapidly to η^5-$C_5H_5Co(CO)L$ in the presence of ligands such as $(C_6H_5)_3P$ or CO.

(8) (a) For details of the electrochemical experiments, see ref 12 in the accompanying communication;[2] (b) an interesting series of paramagnetic, cationic iron dimers having Fe in formal oxidation state +1.5 $[(\eta^5\text{-}C_5H_5)Fe(CO)]_2[Ph_2P(CH_2)_nPPh_2]^+$ has been prepared and studied recently.[8c,d] These complexes are analogous to $\mathbf{1}^{\bullet-}$ in the sense that they contain delocalized adjacent-valent metal atoms; they differ in charge, ligands, and metal–metal bond order (0.5 instead of 1.5); (c) J. A. Ferguson and T. J. Meyer, *Inorg. Chem.*, **11**, 631 (1972); (d) R. J. Haines and A. L. DuPreez, *ibid.*, **11**, 330 (1972).

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(11) National Institutes of Health Postdoctoral Fellow, 1974–present.

(12) National Institutes of Health Postdoctoral Fellow, 1975–present.

(13) Camille and Henry Dreyfus Teacher-Scholar Grant Awardee, 1970–1975.

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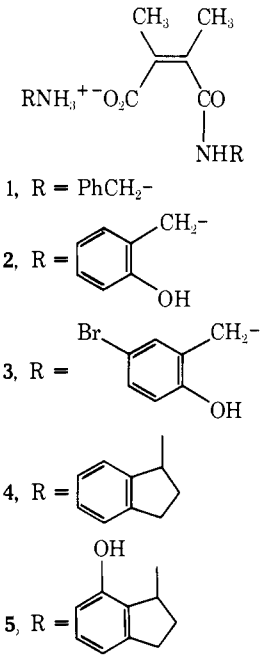
Cooperative Catalysis of the Cleavage of an Amide by Carboxylate and Phenolic Groups in a Carboxypeptidase A Model

Sir:

The enzyme carboxypeptidase A catalyzes the hydrolysis of *N*-acylamino acids and also of the related esters, *O*-acylhydroxy acids, at comparable rates.[1] For both classes of substrates catalytic functions are apparently performed by a Zn^{2+} and a γ carboxylate of glutamate 270. In addition, tyrosine 248 has often been assigned a catalytic role, at least with peptide substrates. It is agreed that the phenolic hydroxyl of Tyr-248 can be within a catalytically useful distance of a bound substrate, and x-ray structure work[2] shows that it can be hydrogen bonded to the leaving group nitrogen in a peptide substrate. However, various modification studies[3] suggest that Tyr-248 plays no role in the hydrolysis of esters, and according to some interpretations[4] may not even be involved in the peptidase activity of the enzyme.

As part of our general program of exploring the chemistry of carboxypeptidase A and various model systems for its action,[5] we have investigated the question of whether the hydrolysis of an amide, catalyzed by a neighboring carboxylate ion, can also be assisted by a phenolic hydroxyl in an appropriate position to protonate the leaving amino group. We find that such bifunctional catalysis is indeed significant, but only under special conditions related to those within the enzyme itself. Perhaps more striking, we find that our reactions undergo a change of mechanism on approach to physiological pH conditions which is directly related to the principal ambiguity in the mechanism of action of carboxypeptidase A.

The compounds of interest, **1–5,** were all prepared by reaction of 2 equiv of the appropriate benzylamine with 2,3-dimethylmaleic anhydride in ether–dimethoxyethane at room temperature for 12 h. Depending on the reaction conditions (vide infra) these compounds underwent cleavage of the amide group to afford either dimethylmaleic anhydride or dimethylmaleic acid. In all cases the kinetics were followed at 250 nm for at least two-three half-lives, and they obeyed a good first-order rate law. In the important pH region corresponding to neutrality, there was no catalysis by buffer, and the identity of the reaction product was confirmed by isolation.



In aqueous solution, all these compounds showed essentially the same behavior as has been described by Kirby[6] for simple *N*-alkyldimethylmaleamic acids. That is, the free carboxylic acid underwent rapid cyclization to produce the dimethylmaleic anhydride, while the corresponding carboxylate ion showed a negligible rate of reaction. Thus, in all aqueous pH ranges compounds **1–5** had similar rates and gave no evidence for catalysis by the phenolic hydroxyl. The situation was different in a nonaqueous medium.

The interior of many enzymes is at least partially nonaqueous in character, and catalytic hydrogen bonding effects do not have to compete with hydrogen bonds involving water. Thus, we have also examined the amide cleavage reactions of compounds **1–5** in CH_3CN containing 1 M H_2O as a model for such a medium. The data are listed in Table I. Of course, the definition of "pH" in such a medium is a problem,[7] so our systems were examined simply in terms of the buffer ratio of HOAc/KOAc. With a 10:1 ratio of HOAc/KOAc, corresponding to an acidic medium, all the compounds underwent a cyclization to produce dimethyl-

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

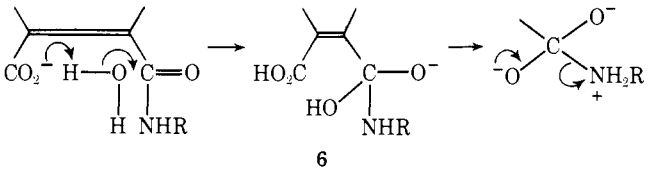
Compd	Mp, °C	10^4k (s^{-1}) with HOAc–KOAc Buffer[a] 10:1	1:10	1:50
1[b]	151–152	75.3	~0.1	0.0314
2[b]	118–120	45.5	1.80	2.07[d]
3[c]	129–136	41.6	5.12	4.55[e]
4[b]	123–125	28.8	~0.07	0.0238
5[c]	107–110	20.1	0.866	0.959

[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.

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(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.
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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.