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Model for the Aspartate Proteinases: Hydrolysis of a Distorted Amide Catalyzed by Dicarboxylic Acids Capable of Forming Cyclic Anhydrides

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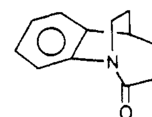
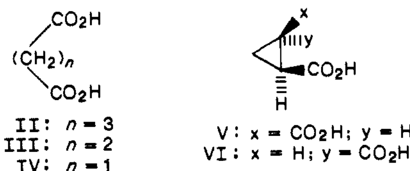
Abstract: A distorted amide (homobenzoquinuclidone, I) is shown to be remarkably susceptible toward attack by dicarboxylic acids capable of forming cyclic anhydrides. The hydrolytic process involves reversible nucleophilic attack to form a linear (open) anhydride. If no second carboxylate is present, or if the remote CO_2H unit is incapable of forming a stable cyclic anhydride, the open anhydride rapidly reverses to re-form I. In the presence of a second carboxylate that is in proximity to the open anhydride, nucleophilic attack occurs to give the amino acid of I and a cyclic anhydride of the diacid. The reaction pH vs log k_2^{obsd} profiles for succinate, glutarate, and *cis*-cyclopropane-1,2-dicarboxylic acid show three domains corresponding to attack of the monoanion of the diacid on I-H^+, monoanion on I, and attack of the dianion on I. The relevance of these observations to the mechanism of hydrolysis of peptides catalyzed by the aspartate proteinases is discussed in terms of the formation of transient anhydride intermediates.

The aspartate proteinases (APases) are widely occurring hydrolytic enzymes containing two essential aspartate residues in the active sites.[1] Included in this class are the digestive enzymes pepsin and chymosin, cathepsin D isolated from lysosomes of many cells, renin, which is the first proteolytic enzyme in the angiotensin cascade, and penicillopepsin from fungi. Alterations in the level of activities of the mammalian enzymes may be associated with pathological conditions such as hypertension (renin), gastric ulcers (pepsin), muscular dystrophy, and neoplastic diseases (cathepsins). X-ray crystallographic determinations[2] have shown close similarities in the active site regions, but the mechanism by which they cleave proteins is still unknown.[3,4] Two main possibilities, nucleophilic or general base/general acid, have been proposed.[1-8] The bulk of the current thinking favors the latter mechanism, primarily because experimental detection of *covalent* intermediates (required in the nucleophilic route) has proven unsuccessful.[4,9]

We recently reported synthetic and physical studies of distorted amide I.[10] That species shows a remarkable reactivity toward nucleophilic attack by bifunctional species such as β-amino al-



cohols[11a] and dicarboxylic acids capable of forming cyclic anhydrides.[11b] In view of the phenomenological similarity between the latter observations[11b] and the cleavage of peptides mediated by the APases, we have extended that study and present the results of the hydrolysis of I catalyzed by acids II–VI.



Experimental

(a) Materials. Amide I was synthesized as described.[10] Malonic, succinic, and glutaric acids were reagent-grade commercial samples (Aldrich) and were used without further purification. *trans*-Cyclopropane-1,2-dicarboxylic acid was prepared as described;[12] mp 174–175 °C (lit.[12] mp 173–174 °C). *cis*-Cyclopropane-1,2-dicarboxylic acid was prepared by hydrolysis of the corresponding anhydride;[13] mp 140 °C (lit.[14] mp 139–140 °C). MOPS (morpholinopropanesulfonic acid) was reagent grade (Sigma). Acetonitrile was twice distilled from P_2O_5 and stored over 3-Å molecular sieves.

(b) Dissociation Constants. The dissociation constants of the various acids employed here were determined at $\mu = 0.3$ (KCl), $T = 25$ °C by potentiometric titration of 0.1 mmol solution of the acid in 6.0 mL of H_2O with 0.01 N NaOH (also at μ 0.3, KCl) using a Radiometer ABU-12 autoburet and Radiometer TTT-2 titrator with a Radiometer GK2321C combination electrode. Values given in Table I are the averages of duplicate measurements.

(c) Kinetics. Triply distilled H_2O was used for all kinetic experiments. Kinetic data were obtained by observing either the rate of increase in absorbance at 291 nm (pH >4.0) or rate of decrease at 250 nm (pH <4.0) of (3–5) × 10^{-4} M solutions of amide I in aqueous media. Reactions were initiated by injecting 5–10 μL of 0.1 M I in CH_3CN into 2–3 mL of buffer. The data were obtained with an HP 8451A diode array spectrophotometer. Throughout the useful buffering range of the

(1) (a) *Aspartic Proteinases and Their Inhibitors*; Proceedings of FEBS Advanced Course No. 84/07; Kosta, V., Ed.; Walter de Gruyer: Berlin, 1985. (b) Tang, J., Ed. *Acid Proteinases, Structure, Function, and Biology*; Plenum: New York, 1977; p 95. (c) Fersht, A. *Enzyme Structure and Mechanism*; W. H. Freeman: New York, 1985; pp 422–426. (d) Fruton, J. P. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1976**, *44*, 1–36. (e) Polgár, L. *FEBS Lett.* **1987**, *219*, 1–4.

(2) (a) Hsu, I.-N.; Delbaere, L. T. J.; James, M. N. G.; Hofmann, T. *Nature (London)* **1977**, *266*, 140–145. (b) Subramanian, E.; Swan, I. D. A.; Liu, M.; Davies, D. R.; Jenkins, J. A.; Tickle, I. J.; Blundell, T. L. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 556–559. (c) Jenkins, J. A.; Tickle, I. J.; Sewell, T.; Ungaretti, J.; Wollmer, A.; Blundell, T. In *Acid Proteases, Structure, Function and Biology*; Tang, J., Ed.; Plenum: New York, 1977; pp 43–60. (d) Andreeva, N. S.; Zdanov, A. S.; Gustchina, A. E.; Fedorov, A. A. *J. Biol. Chem.* **1984**, *259*, 11353–11365. (e) Bott, R.; Subramanian, E.; Davies, D. R. *Biochemistry* **1982**, *21*, 6956–6962. (f) James, M. N. G.; Sielecki, A. R. *Biochemistry* **1985**, *24*, 3701–3713 and references therein.

(3) For a review on the current status of thinking on the mechanism, see: Appendix of Hofmann, T.; Dunn, B. M.; Fink, A. L. in ref 4a.

(4) (a) Hofmann, T.; Fink, A. L. *Biochemistry* **1984**, *23*, 5247–5256. (b) Dunn, B. M.; Fink, A. L. *Biochemistry* **1984**, *23*, 5231–5247.

(5) Knowles, J. R. *Philos. Trans. R. Soc. London, B* **1970**, *257*, 135–146.

(6) (a) Aldersley, M. F.; Kirby, A. J.; Lancaster, P. W.; McDonald, R. S.; Smith, C. R. *J. Chem. Soc., Perkin Trans. 2* **1974**, 1487–1495. (b) Kirby, A. J.; McDonald, R. S.; Smith, C. R. *Ibid.* **1984**, 1495–1504.

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(9) Transpeptidation studies that are interpreted in terms of intermediates being generated and held by the enzyme long enough for a second substrate to be bound and peptide re-formation to occur have been reported: Blum, M.; Cunningham, A.; Bendiver, M.; Hofmann, T. *Biochem. Soc. Trans.* **1985**, *13*, 1044–1046. This same possibility has been suggested by Kluger and Chin.[7] Such intermediates may indeed by bound but not necessarily covalently linked to the enzymes at all times.

(10) Somayaji, V.; Brown, R. S. *J. Org. Chem.* **1986**, *51*, 2676–2686.

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(13) We are grateful to Dr. K. Krowicki, Department of Chemistry, University of Alberta, for a gift of the anhydride.

(14) Wasserman, A. *Helv. Chim. Acta* **1903**, *13*, 223–226.

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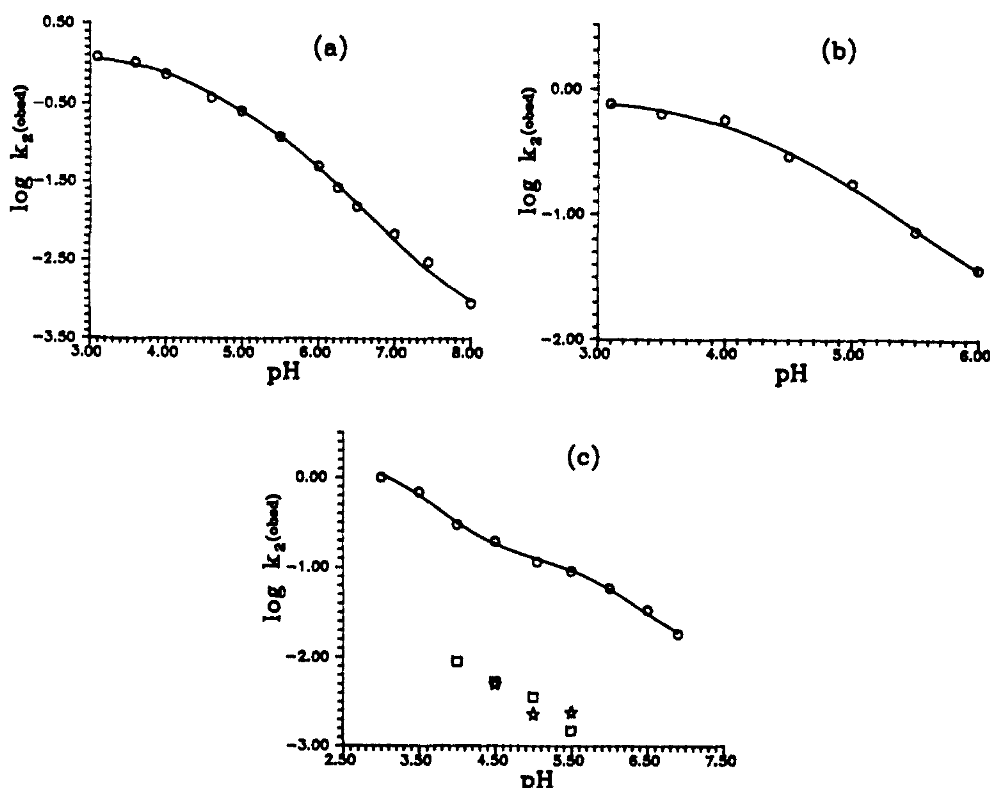


Figure 1. Plots of log k_2^{obsd} vs pH for various diacids with I, $T = 25$ °C, $\mu = 0.3$ (KCl). Key: (a) succinate; (b) glutarate; (c) *cis*-cyclopropane-1,2-dicarboxylic acid (○), malonate (☆), *trans*-cyclopropane-1,2-dicarboxylic acid (□).

diacids (pH values between $pK_{a_1} - 1$ and $pK_{a_2} + 1$), buffers of the diacid alone were used with the ionic strength being maintained at 0.3 (KCl). Above $pK_{a_2} + 1$, buffers consisting of dilute solutions of MOPS buffer (0.1 M at pH 7.00, 0.05 M at pH 7.45 and 8.0) were used in conjunction with the diacids. In no cases did the pH values before and after a kinetic run vary more than 0.02 unit. Pseudo-first-order rate constants (k_{obsd}) for the disappearance of I were determined by fitting the A vs time data to a standard exponential model. Second-order rate constants (k_2^{obsd}) were obtained as the slopes of the linear regression analysis of the k_{obsd} values at a given pH vs [diacid] (6–12 points, 3–4 concentrations, r >0.999). In no case was biphasic behavior observed in the k_{obsd} measurements, and no saturation kinetics were observed in the k_{obsd} vs [diacid] plots. Given in Tables 1S–3S (supplementary material) are the second-order rate constants at various pH values for succinic, glutaric, and *cis*-cyclopropane-1,2-dicarboxylic acids.

(d) FTIR Studies. IR spectra (2200–1600 cm^{-1}) of acetonitrile solutions of I and the acids (0.1 M in each) were measured (KBr cell, 0.1-mm path length) on a Nicolet 7199 FTIR spectrophotometer. The following is a representative procedure. A 0.1 M triethylammonium hydrogen succinate solution was prepared by dissolving 1.0 mmol (118 mg) of succinic acid and 1.0 mmol (101 mg, 139 µL) of triethylamine in 10 mL of CH_3CN. The KBr cell was filled with CH_3CN alone, and the background spectrum was recorded and used as a reference to be subtracted from future spectra. Amide I (1.87 mg, 0.01 mmol) was weighed into a dry vial and dissolved in 100 µL of the above triethylammonium hydrogen succinate solution. The solution was quickly transferred to the KBr cell and scanning initiated. The scans (~60/min) were averaged every 10 min over a period of 16 h. The spectra were then corrected for the background by subtracting the reference from the 10-min-averaged spectra.

(e) ^{1}H NMR. ^{1}H NMR experiments were recorded with a Bruker WP-80 spectrometer. One set of experiments was performed with amide and acetate (CD_3CN solvent) to establish the formation of an open anhydride. The aromatic protons of I appear as a close multiplet at δ 7.25 (4 H), while those of the corresponding amino acid or ethyl ester appear at δ 7.00 (m, 2 H) and 6.55 (m, 2 H). Assuming this to be the case for the open anhydride as well, it should be possible to gauge the extent of acetate attack on I.

Amide I (18 mg, 0.1 mmol) was dissolved in 400 µL of CD_3CN and the spectrum recorded. Triethylamine (101 mg, 139 µL, 1 mmol) and CD_3COOD (64 mg, 56 µL, 1 mmol) were added, and the spectrum was recorded after 5 and 15 min. The procedure was repeated after the addition of a second 1-mmol portion of each of the latter two reagents. A substantial amount of the open anhydride was formed, but ~20% of the starting amide was still visible at equilibrium. At this stage, the solution consisted of a 20:1 ratio of acetate/amide.

A small amount of the above slution was analyzed by CI mass spectrometry. An intense peak was observed at m/z 251, corresponding to the open anhydride ($C_{14}H_{14}D_3NO_3 + H^+$). Another 25-µL portion of the above solution was injected into 3.0 mL of 0.1 M acetate buffer (pH 4.75, $\mu = 0.3$, $T = 25$ °C) and the UV spectrum immediately monitored. In that case, an immediate formation of I (<6 s) was observed followed by its subsequent normal hydrolysis.[15]

Table I. pK_a Values of Various Acids Determined by Potentiometric Titrations [$T = 25$ °C, $\mu = 0.3$ (KCl)][a]

acid	pK_{a_1}	pK_{a_2}
acetic	4.55	
malonic	2.55	5.10
succinic	4.05	5.25
glutaric[b]	3.98	4.97
cis-cyclopropane-1,2-dicarboxylic	3.10	5.84
trans-cyclopropane-1,2-dicarboxylic[b]	3.39	4.58

[a] Average of two determinations, ±0.03 unit. [b] Corrected as in ref 16.

Results and Discussion

(a) Kinetics and pK_a Values. Although the various pK_a values for all the acids in this study are available in the literature, we required values at $T = 25$ °C, $\mu = 0.3$, in order to correlate them with the kinetic experiments. Given in Table I are the values from the duplicate potentiometric titrations. In cases where the pK_a values for a given diacid are separated by <1.7 units, they were corrected according to a published procedure.[16]

Shown in Figure 1a–c are plots of the log second-order rate constant (k_2^{obsd}) vs pH profiles for the disappearance of I mediated by succinic (III), glutaric (II), and *cis*-cyclopropane-1,2-dicarboxylic (V) acids. (The individual rate constants are given in Tables 1S–3S, supplementary material.) Also shown in Figure 1c are the log k_2^{obsd} vs pH profiles for the same process catalyzed

(15) $k_{obsd} = 1.64 \times 10^{-3}$ s^{-1}; for authentic I under the identical conditions, $k_{obsd} = 1.70 \times 10^{-3}$ s^{-1}.

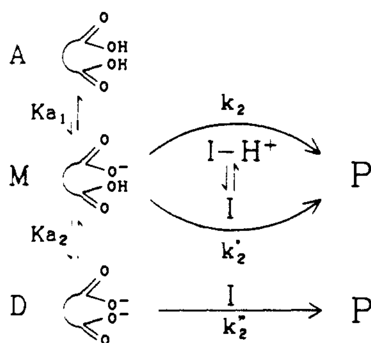
(16) Albert, A.; Serjeant, E. P. *Ionization Constants of Acids and Bases*; Wiley: New York, 1962; p 52.

Table II. Values for the Constants Given in Scheme I Derived from Nonlinear Least-Squares Fits of k_2^{obsd} vs $[H^+]$[a]

constant, M^{-1} s^{-1}	glutaric acid (II)	succinic acid (III)	*cis*-cyclopropane-1,2-dicarboxylic acid (V)
k_2/K_a[b]	$(7.77 \pm 0.45) \times 10^3$	$(1.35 \pm 0.09) \times 10^4$	$(2.33 \pm 0.13) \times 10^3$
k_2'[c]	$(2.58 \pm 0.25) \times 10^{-1}$	$(2.72 \pm 0.14) \times 10^{-1}$	$(1.22 \pm 0.08) \times 10^{-1}$
k_2''	$(1.42 \pm 0.45) \times 10^{-2}$	$(4.18 \pm 1.02) \times 10^{-4}$	$(9.75 \pm 2.18) \times 10^{-3}$

[a] k_2^{obsd} values given in Tables 1S–3S (supplementary material), T = 25 °C, μ = 0.3 (KCl); error limits from standard deviations of the fits. [b] Ka defined as the acid dissociation constant of I-H$^+$. [c] Note kinetic ambiguity since attack of M on I is equivalent to D on I-H$^+$.[19]

Scheme I



by malonic (IV) and *trans*-cyclopropane-1,2-dicarboxylic (VI) acids (Table 4S, supplementary material.) From a cursory comparison of the profiles, it can be seen that the diacids capable of forming cyclic anhydrides are 1–2 orders of magnitude more efficacious in promoting the decomposition of I than are malonic or the trans cyclopropane acids.

The data for diacids II, III, and V can be described by the simplified process given in Scheme I. From that, the rate of disappearance of amide is given by eq 1, where A, M, and D are

$$-d[I]_{tot}/dt = k_2[I\text{-}H^+][M] + k_2'[I][M] + k_2''[I][D] \quad (1)$$

the acidic, monoionic, and dianionic forms of the diacid and I and I-H$^+$ are the neutral and protonated forms of the amide. Since $[I]_{tot} = [I] + [I\text{-}H^+]$ and at all pH values $[H^+] \ll K_a$,[17] eq 1 yields eq 2. Given that the various forms of the diacid are also pH

$$\frac{-d[I]_{tot}}{dt} = [(k_2/K_a)[H^+][M] + k_2'[M] + k_2''[D]][I]_{tot} \quad (2)$$

dependent and $[\text{diacid}]_{tot} = [A] + [M] + [D]$, it can be shown that

$$-d[I]_{tot}/dt = k_2^{obsd}[I]_{tot}[\text{diacid}]_{tot} \quad (3)$$

where

$$k_2^{obsd} = \frac{(k_2/K_a)K_{a_1}[H^+]^2 + k_2'K_{a_1}[H^+] + k_2''K_{a_1}K_{a_2}}{[H^+]^2 + K_{a_1}[H^+] + K_{a_1}K_{a_2}} \quad (4)$$

Nonlinear least-squares fitting of the k_2^{obsd} data for the diacids II, III, and V against $[H^+]$ yields the values for the various constants given in Table II, the K_{a_1} and K_{a_2} values being known from potentiometric titration. The lines through the data points in Figure 1a–c are those calculated in accordance with eq 4. Inclusion of the term corresponding to the acid dianion (D) attacking I appears justified since without the term the fits noticeably deviate downward from the data points at high pH. However, because the reactions are slow at the high pH end, and the effect of the diacid is small, we were unable to obtain good rate data.

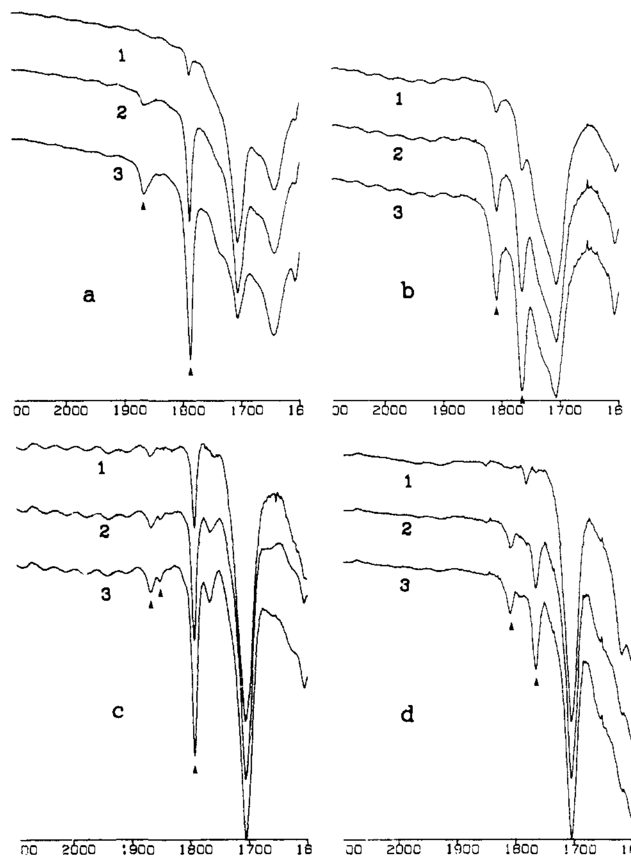


Figure 2. FTIR spectra of monotriethylammonium salt of diacids with equimolar (0.1 M) I in CH_3CN at various times showing the buildup of cyclic anhydrides. Spectra: 1, T = 10 min; 2, $T \simeq$ 3 h; 3, $T \simeq$ 16 h. Anhydride bands in each case are marked with (↑). Key: (a) succinate; (b) glutarate; (c) *cis*-cyclopropane-1,2-dicarboxylic acid; (d) maleate.

As a consequence, there is at least a 25% uncertainty in k_2''. Although the involvement of the monoanion (M) with the protonated form of the amide (I-H$^+$) at low pH, and D with I at high pH seems relatively certain, there is a mechanistic ambiguity in the middle-pH range. Two kinetically equivalent possibilities exist, namely M + I (which is shown in Scheme I) or D + I-H$^+$.[19] There is no unambiguous distinction; however, we prefer the former process since it is not clear why the $(k_2/K_a)/(k_2'/K_a)$[19] ratio (Table II) would be as large as $(2-5) \times 10^4$ for a monoanion relative to a dianion attacking I-H$^+$.

(b) Intermediates. During the kinetic studies in H_2O, evidence such as biphasic or saturation kinetics, which would be suggestive of the formation of intermediates, was not observed in any case. This could indicate that either the various forms of the diacid function as general-acid/general-base catalysts or, if intermediates are formed, their rate of decomposition is very fast.

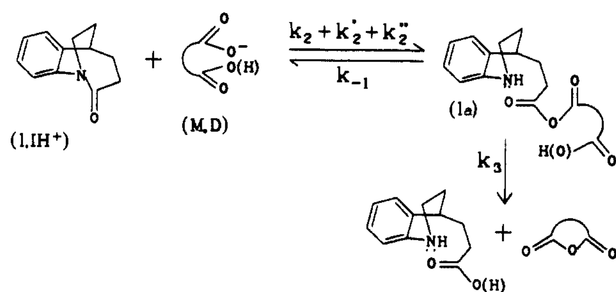
More definitive evidence for the involvement of intermediates comes from FTIR studies of 0.1 M I in CH_3CN containing 1 equiv of the $Et_3^+NH^-O_2CRCO_2H$ salts of II, III, V, and maleate.

(17) Normally, simple N-protonated amides have pK_a values of −8 to −6,[18] while O-protonated amides have pK_a values on the order of −2 to 0.[18] The relative acidities can usually be rationalized on the basis of resonance arguments. However, because of the distorted nature of I[10,11a] the "normal" amide resonance is diminished so that pK_a values of the NH$^+$ and C=OH$^+$ forms should tend to approach each other. While we do not know where the proton is located in I-H$^+$, a first estimate of its pK_a is ∼−3 to −5.

(18) (a) Arnett, E. M. *Prog. Phys. Org. Chem.* **1963**, *1*, 223–403. (b) Guthrie, P. J. *J. Am. chem. Soc.* **1974**, *96*, 3608–3615. (c) Arnett, E. M.; Quirk, R. P.; Larsen, J. W. *Ibid.* **1970**, *92*, 3977–3984.

(19) In Scheme I if the process is actually involved D + I-H$^+$, eq 4 would appear as $k_2^{obsd} = [(k_2/K_a)K_{a_1}[H^+]^2 + (k_2'/K_a)K_{a_1}[H^+] + k_2''K_{a_1}K_{a_2}]/([H^+]^2 + K_{a_1}[H^+] + K_{a_1}K_{a_2})$. Hence, the k_2' value in Table II would actually be k_2'/K_a. It is also possible in the mid pH range that both processes (M + I; D + I-H$^+$) occur simultaneously.

Scheme II



Shown in Figure 2a–d are the IR spectra of I with each monocarboxylate taken at T = 10 min and 3 and 16 h. For each of these diacids, the C=O stretching bands of the cyclic anhydride[20] are seen to grow at the expense of the initial C=O band of I. On the other hand, with diacids where no cyclic anhydride can be formed, (e.g., malonate, acetate) no change in the spectrum is detected over a 16-h period. Control experiments establish that $Et_3{}^+NH^-O_2CRCO_2H$, either alone or in the presence of equimolar authentic amino acid of I, shows no tendency to form anhydrides. Also, in no case with equimolar (0.1 M) reagents is a linear anhydride detected.

Finally, we have looked by ^1H NMR at the reaction of 0.25 M I with excess $Et_3{}^+ND^-O_2CCD_3$ in CD_3CN. When the acetate/I ratio is ~20/1, integration of the aromatic region shows an approximate 4/1 ratio in favor of the open anhydride. The presence of that species is also verified by CI mass spectrometric analysis of the NMR mixture. Finally, when an aliquot of the NMR mixture is rapidly injected into 3.0 mL of pH 4.75, 0.1 M acetate buffer (T = 25 °C) and the UV spectrum immediately recorded, only the starting amide could be detected followed by its normal rate of hydrolysis in that medium.

The picture that emerges is that given in Scheme II wherein a carboxylic acid can nucleophilically attack I to yield an open anhydride (Ia), which in the absence of a suitably positioned second carboxylate rapidly reverses. If we assume from the above acetate UV experiment that the $t_{1/2}$ for regeneration of I from its open anhydride is ≤1 s, then the rate constant for reclosure to form I is ≥0.7 s^{-1}. The rates of reversal for all the acids investigated are expected to be similar so that nucleophilic attack by carboxylate alone does not lead to such enhanced hydrolysis.[21] However, in the cases where a suitably positioned second carboxylate is present, an additional reaction pathway is available to Ia that leads to hydrolysis and a cyclic anhydride. In those cases it is not yet known whether k_3 is sufficiently large to suppress reformation of I completely. Intramolecular nucleophilic attack by carboxylates to form five and six-membered cyclic anhydrides is well-known to be an effective pathway for the hydrolysis of succinate, maleate, and glutarate half-esters and amides.[22a] This appears to be particularly effective in the present system, since no evidence for the open anhydride (Ia) exists in the reaction of I with II, III, and V.

The lack of reactivity of malonate, for which a four-membered anhydride is too strained,[22b] also supports the kinetic involvement of the above pathway and not a significant general-acid/base catalyzed decomposition of Ia by the remote CO_2H unit. Although general-base catalysis of anhydride hydrolysis by carboxylates is known[22] and molecular models suggest it could structurally be accommodated in Ia formed with malonate, the process is apparently too inefficient to compete with reversal to I.

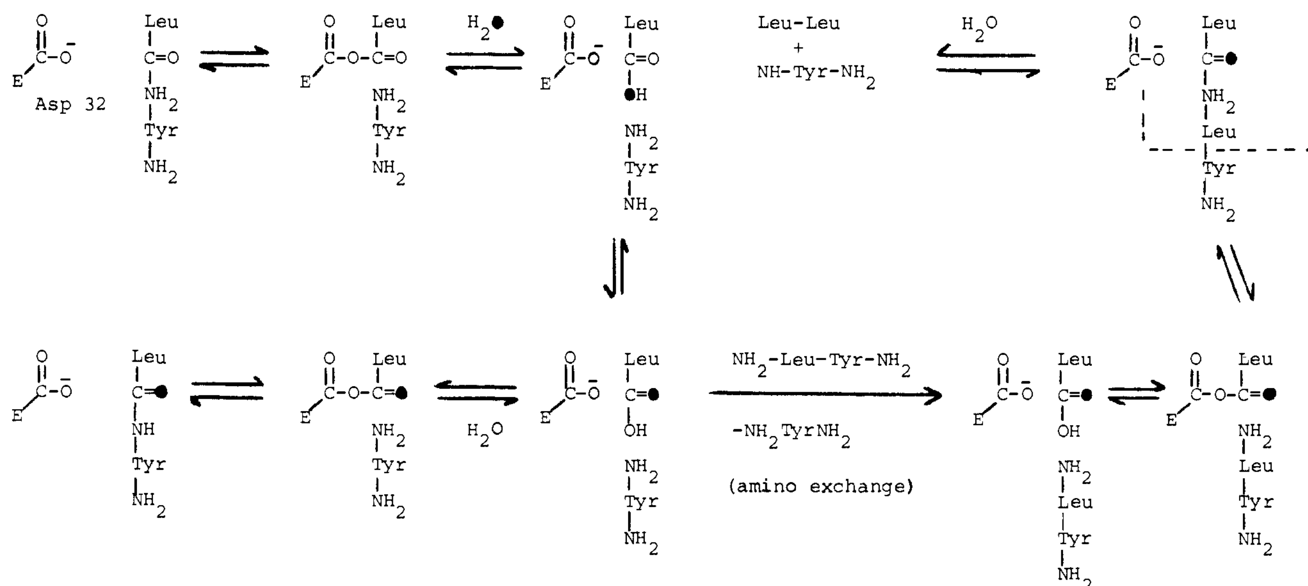
Conclusions and Relevance to the APases

Amide I is unique in that structural distortion increases its susceptibility to nucleophilic attack by β-amino alcohols[11a] and certain dicarboxylic acids.[10] From this study, the pathway for the latter reaction clearly proceeds via formation of anhydride intermediates. Formally, the hydrolysis occurs without water per se, since its constituents are created stepwise by the conversion of the attacking diacid into a cyclic anhydride. The inherent distortion in I provides the driving force for this reaction.

Substrate distortion has been considered an important aspect of the APase-catalyzed hydrolysis of peptides.[24] Although one may debate[25] whether the enzyme distorts the peptide as much, or in the same fashion as in I, there is little doubt that any substrate distortion that would diminish the amide resonance should enhance the attack of nucleophiles [H_2O or $CO_2(H)$] on the acyl unit.[26] For the APases, the bulk of current thinking favors a general-base/general-acid role for the active site Asp units, primarily because attempts to detect covalent intermediates by trapping or spectroscopic means have so far proven unsuccessful. Nevertheless, the fact that pepsin and penicillopepsin promote trans-peptidations strongly implies the formation of tightly bound or covalent intermediates.[3,9,27] It is of note that although the catalyzed decomposition of I must proceed via an intermediate linear anhydride, that species is not detectable in aqueous solution because its decomposition is much faster than formation. As pointed out by Hofmann, Dunn, and Fink,[3,4a] such a possibility exists for the enzymes as well.

On the other hand, Antonov[28] has reported studies in $H_2{}^{18}O$ that indicate that during the course of Leu-Tyr-NH_2 hydrolysis no ^{18}O is incorporated into the enzyme active site aspartates. However, ^{18}O is incorporated into the Leu-Leu transpeptidation

Scheme III. Proposed Pathway for ^{18}O-Exchange into Recovered Leu-Tyr-NH_2 and Leu-Leu Transpeptidation Product Catalyzed by Pepsin (●, ^{18}O)



product to the extent of ~60% as well as ~10% into recovered Leu-Tyr-NH_2 substrate.[29] Antonov has concluded that the fact ^{18}O is incorporated into the substrate "confirms the conclusion that pepsin catalysis follows the general-base mechanism".[28,29] There is however a mechanism, (Scheme III), which is a modification of that given by Hofmann, Dunn, and Fink,[4a] that would explain the above results and involves a transient anhydride intermediate that is rapidly cleaved by $H_2{}^{18}O$ specifically at the Leu C=O unit.[30] If the Leu CO_2H oxygens can tautomerically and positionally equilibrate, then such a process could account for the ^{18}O in the substrate and product and moreover does not place ^{18}O in the Asp-32 unit, since the same oxygen that attacks the Leu C=O is the one that departs. If we accept the Antonov results that indicate no ^{18}O is incorporated into the enzyme Asp units, then subsequent formation of an $Asp_{32}C(O)OC(O)Asp_{215}$ anhydride cannot occur since, in order for the enzyme to turn over, at least one $H_2{}^{18}O$ must be incorporated. If this is so, then the relevance of the subsequent formation of cyclic anhydrides from Ia to the enzymatic process is questionable. Nevertheless, the occurrence of the latter process in the hydrolysis of I does not invalidate a nucleophilic role for carboxylate in the enzyme.

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Supplementary Material Available: Tables of second-order rate constants at various pH values for diacids II–VI with I, $T = 25$ °C, $\mu = 0.3$ (KCl) (4 pages). Ordering information is given on any current masthead page.

(20) The presence of succinic and glutaric anhydride was verified by comparison of the C=O stretching frequencies of authentic materials. In the case of the anhydride of *cis*-cyclopropane-1,2-dicarboxylic acid, FTIR analysis of authentic material confirms that the high-frequency band is a doublet at 1851 and 1868 cm^{-1}.

(21) As can be judged from Figure 4, acetate, malonate, and *trans*-cyclopropane-1,2-dicarboxylate are catalysts for the hydrolysis of I, but their mode of action is at present unknown.[6,10]

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(30) Extending this mechanism, it is now possible to explain why Antonov observed more than the expected 50% ^{18}O-incorporation in Leu-Leu.[29] If the initially cleaved anhydride (with one ^{18}O in Leu-COOH) re-forms the anhydride to a certain extent and is again cleaved by $H_2{}^{18}O$, then larger amounts than 50% are to be expected. Also, from this scheme it is possible to explain how the enzyme can act as an acyl-transfer or amino-transfer catalyst simply by postulating that either the initially produced acid or amine can leave the active site while its partner is retained.

Time-Resolved Proton–Phospholipid Interaction. Methodology and Kinetic Analysis

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Abstract: The proton transfer between bulk and phospholipid surface was measured at real time by the laser induced proton pulse method. (Gutman, *Methods Biochem. Anal.* **1984**, *30*, 1–103). A pH indicator was adsorbed either on phospholipids–Brij-58 mixed micelles or on small unilamelar liposomes, and the protonation dynamics were recorded. The probe reactions are sensitive to the composition of the surface; its rate and extent of protonation vary with the surface density of the phospholipids and their p*K*. The observed transients were analyzed by numerical solution of coupled differential equations. The solution determines the rate constants of the phosphohead groups protonation and the rate they exchange protons with the probe. Through this analysis we can account for the capacity of the membrane to function as proton-collecting anthena; the protons first react with the acidic, ionized moieties on the surface and then, by rapid exchange, reach the strongest base on the surface. Both trapping capacity and rate of flux between surface groups are affected by the p*K*. Proton trapping is enhanced by less acidic lipids like phosphatidylserine (p*K* = 4.6). Proton mobility is enhanced by more acidic groups like phosphatidylcholine (p*K* = 2.2). This analysis predicts how combination of the two effects will determine apparent reactivity of a probe on phospholipid membrane.

The surface of the biomembranes is where most proton-transfer reactions take place. This region was in the center of interest for many years, but still we have very little information concerning the kinetic parameters associated with proton diffusion on the surface or between surface and bulk.

The understanding of the mechanism of proton transfer must stem from time resolved observations of proton diffusion between defined source and sink located in the precise position with respect to the membrane. The turnover of protogenic enzymes, like ATPase, is measured in milliseconds. Such enzymes are too slow

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