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On the Possible Involvement of an Anhydride Intermediate in Papain-Catalyzed Hydrolyses*

Donald C. Williams and John R. Whitaker

ABSTRACT: The papain-catalyzed hydrolysis of *p*-nitrophenyl benzyloxycarbonylglycinate was carried out in $H_2{}^{18}O$, and the papain was isolated from the reaction and allowed to react with more of the substrate in $H_2{}^{16}O$. Benzyloxycarbonylglycine isolated from the reaction in $H_2{}^{18}O$ was labeled with ^{18}O to the same extent as the ^{18}O content of the solvent; however, benzyloxycarbonylglycine isolated from the second hydrolysis in $H_2{}^{16}O$ was not enriched in ^{18}O. In a model system, *o*-mercaptobenzoic acid was used to catalyze the hydrolysis of *p*-nitrophenyl benzoate in $H_2{}^{18}O$. While the benzoic acid isolated from the reaction mixture was enriched in ^{18}O to nearly the extent of the ^{18}O content of the solvent, there was no ^{18}O enrichment of *o*-mercaptobenzoic acid. These data suggest that neither of the catalyzed reactions involve an anhydride intermediate. The essential carboxyl group in papain and the carboxyl group of *o*-mercaptobenzoic acid probably function as a general base in the catalyses.

On the basis of the pH–activity profiles, heats of ionization, and chemical inhibition studies, it has been suggested that the activity of papain is dependent upon a carboxyl group and a sulfhydryl group in the active center (Stockell and Smith, 1957; Smith *et al.*, 1958). These studies have been confirmed and extended (Sanner and Pihl, 1963; Sluyterman, 1964; Whitaker and Bender, 1965; Bender and Brubacher, 1966). Recently, it was indicated that these groups may be cysteine residue 25 (the only cysteine residue in papain) and aspartic acid residue 163 (Light *et al.*, 1964).

The function of the sulfhydryl group as acceptor of the acyl portion of the substrate molecule has been indicated by isolation and spectrophotometric observation of the acyl-enzyme intermediate (Lowe and Williams, 1964; Bender and Brubacher, 1964; Brubacher and Bender, 1966). The function of the carboxyl group in the mechanism of action of papain and related enzymes is not clear. Bender and collaborators (Bender, 1957; Bender *et al.*, 1958; Schonbaum and Bender, 1960) have studied some intramolecular catalyzed reactions which were postulated to simulate the action of the sulfhydryl proteolytic enzymes. In these models, it was proposed that the carboxylate group participates as a nucleophile to accept the acyl moiety from the sulfhydryl group so as to form an anhydride intermediate. On the basis of these models it has been suggested that such an

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anhydride intermediate may be formed in the hydrolysis of substrates by ficin and papain (Hammond and Gutfreund, 1959; Bender, 1960a,b; Ingraham, 1962).

In the case of ficin and papain there is no direct evidence to indicate the formation of an anhydride intermediate. While one would not expect to observe the formation of a transient anhydride intermediate spectrophotometrically or to isolate it, evidence for such an intermediate might be obtained by the use of ^{18}O-enriched water. This paper reports the results of hydrolyses performed in ^{18}O-enriched water in which papain (enzyme system) and *o*-mercaptobenzoic acid (model system) served as catalysts. There was no incorporation of ^{18}O into the catalyst.

Materials and Methods

Materials. Papain (Worthington Biochemical Corp., lot PAP 7DB, two-times crystallized) was recrystallized as the mercuripapain derivative (Brubacher and Bender, 1966). Just before use, the crystals of mercuripapain were harvested by centrifugation, dissolved in 3.33 ml of 10.00% ^{18}O-enriched water (Bio-Rad Laboratories, lot 82324, 1.16 atom % deuterium), and activated with 4-methylbenzenethiol (Soejima and Shimura, 1961) as described by Williams and Whitaker (1967). The normality of the papain solution was determined using α-*N*-benzoyl-L-arginine ethyl ester (BAEE)[1] and the correlation between the rate assay with BAEE and titration assay with *p*-nitrophenyl benzyloxycarbonyl-L-tyrosinate (*p*-nitrophenyl Z-L-tyrosinate) reported by Bender *et al.* (1966).

p-Nitrophenyl benzoate was synthesized according to the method of Chattaway (1931) except we used benzoyl chloride rather than benzoic anhydride. The melting point of the ester was 144.5–145.5° (Fisher melting point block) (lit. (Cilento, 1953) mp 145–146°). *o*-Mercaptobenzoic acid (Sigma Chemical Co.) was used without recrystallization (mp 165.0-165.5°, lit. (Schonbaum and Bender, 1960) mp 164°). 5,5′-Dithiobis(2-nitrobenzoic acid) was from K & K Laboratories, Inc. (lot no. 6273). Acetonitrile was from Eastman. *p*-Nitrophenyl benzyloxycarbonylglycinate (*p*-nitrophenyl Z-glycinate) was from Sigma Chemical Co. (lot C111B-77). Sephadex G-25 was from Pharmacia Fine Chemicals Inc. All other compounds were reagent grade and deionized water was used.

Papain-Catalyzed Hydrolysis. To 82.8 mg of activated papain (70.5% enzymatically active) in 3.33 ml of ^{18}O-enriched water was added 12.9 mg of *p*-nitrophenyl Z-glycinate (molar ratio of substrate to active enzyme of 14.0). The reaction was maintained at room temperature (approximately 25°) and at pH 5.2 by addition of 0.283 N NaOH from a Radiometer pH-Stat (Titrator 11, Titragraph SBR 2c, pH meter 25, buret SBUla). The theoretical amount of base was consumed. Immediately on completion of reaction, the reaction mixture was separated on a Sephadex G-25 column (1.7 × 25.0 cm) equilibrated with 0.1 M triethylamine acetate buffer (pH 5.2). The papain, Z-glycine, and *p*-nitrophenol were completely separated, the first on basis of size, the latter two because of retardation due to adsorption of *p*-nitrophenol on the column.

The separated enzyme (in 15 ml of 0.1 M triethylamine acetate buffer (pH 5.2) made with nonenriched water) was immediately reactivated with 4-methylbenzenethiol. To 60.0 mg of activated papain (76.6 % enzymatically active) was added 3.94 mg of *p*-nitrophenyl Z-glycinate (molar ratio of substrate to active enzyme of 5.45). The reaction was permitted to go to completion and the reaction mixture was separated on Sephadex G-25 as above. Those fractions containing Z-glycine were lyophilized, redissolved in water, and lyophilized again. This was repeated several times to remove the majority of the triethylamine acetate buffer. The residue was acidified with a minute amount of concentrated HCl and repeatedly extracted with ether. The ether was dried over a few granules of anhydrous sodium sulfate, the sodium sulfate was removed, and the ether was evaporated to dryness. The crystalline residue was further dried over P_2O_5 *in vacuo*. Analysis for ^{18}O enrichment of Z-glycine was performed in a Varian M66 mass spectrometer. Repetitive scans of the mass range of 207–212 (expanded to cover full chart length) were made for each sample. Scans of 209–210 and of 210–211 were made in order to utilize 60–70% maximum scale height for 209 and 210, respectively. Areas under the curves were cut out and weighed to permit calculation of the per cent contribution of masses 209, 210, and 211 in Z-glycine.

o-Mercaptobenzoic Acid Catalyzed Hydrolysis. The reaction mixture contained 31.6 mg (0.130 mmole) of *p*-nitrophenyl benzoate, 20.1 mg (0.130 mmole) of *o*-mercaptobenzoic acid, 0.01 ml of 3.3×10^{-2} M Versene (0.123 mg, 3.3×10^{-4} mmole), 113.8 mg of Tris-HCl buffer (final concentration, 0.28 M), 1.4 ml of acetonitrile (final concentration, 30%), 3.3 ml of 10.00% ^{18}O-enriched water, and 0.02 ml of concentrated hydrochloric acid to bring the reaction mixture to exactly pH 8.0. The reaction was carried out at 74° (the reaction pH at 74° was 7.2) for 10 hr under nitrogen (to retard oxidation of the *o*-mercaptobenzoic acid) in a 10-ml ground-glass-stoppered volumetric flask. The temperature was maintained by a rheostat-controlled heating jacket filled with glass beads. The final pH of the reaction mixture was 7.7 (25°). The reaction was shown to be complete by *p*-nitrophenol analysis (see below).

Benzoic acid and *o*-mercaptobenzoic acid were isolated in the following way. The reaction mixture was acidified to pH 5.67 and the mixture was repeatedly extracted with ether to remove the *p*-nitrophenol. After adjustment of the pH to approximately 2, benzoic acid and *o*-mercaptobenzoic acid (and any of the disulfide of mercaptobenzoic acid formed by oxidation) were extracted with ether. The ether was removed over a steam bath and the residue was extracted into 2.0 ml of 0.1 M acetate buffer (pH 6.0). Any disulfide formed was reduced by mixing and shaking vigorously for 20 min with 40 ml of a toluene solution containing 4.5×10^{-2} M 4-methylbenzenethiol. The aqueous layer was filtered on

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: BAEE, α-*N*-benzoyl-L-arginine ethyl ester; Z, benzyloxycarbonyl.

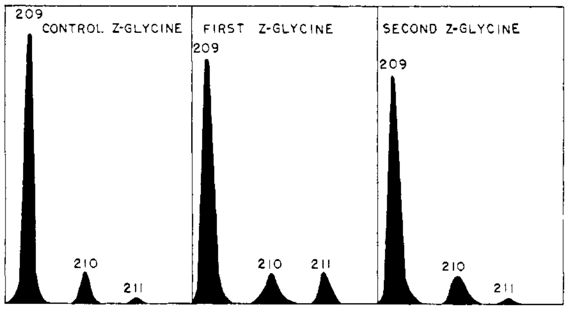


FIGURE 1: Mass spectrometric analysis of Z-glycine. The parent fragments of mass 209, 210, and 211 as determined on an expanded scale are shown. The areas under the curves are relative within a sample only.

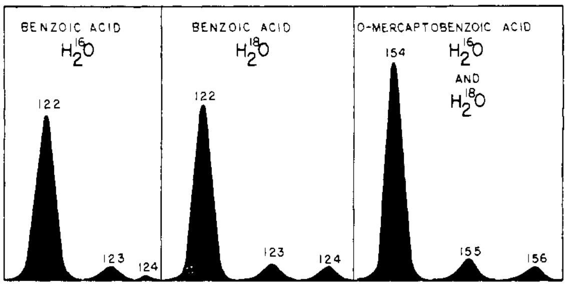


FIGURE 2: Mass spectrometric analysis of benzoic acid and *o*-mercaptobenzoic acid. The parent fragments of mass 122, 123, and 124 and mass 154, 155, and 156 for benzoic acid and *o*-mercaptobenzoic acid, respectively, as determined on an expanded scale are shown. The areas under the curves are relative within a sample only. The mass composition of *o*-mercaptobenzoic acid was exactly the same when the reactions were performed in either $H_2{}^{16}O$ or $H_2{}^{18}O$.

filter paper to remove traces of toluene. Water (2 ml) containing mercuric chloride (100 mg) was added to the aqueous solution and the mixture was shaken vigorously. The precipitated *o*-mercaptobenzoic acid (as the mercuri derivative) was separated from the supernatant liquid by centrifugation.

The supernatant liquid was acidified to pH 2 and the fine white precipitate of benzoic acid was separated from the supernatant liquid by centrifugation. The precipitate was extracted with ether, the ether was removed by evaporation, and the residue was recrystallized from alcohol–water and dried *in vacuo* over P_2O_5 (mp 120–124°).

The mercuri derivative of *o*-mercaptobenzoic acid was suspended in 2.0 ml of 0.1 M acetate buffer (pH 6.0) and hydrogen sulfide was bubbled through the suspension for 45 min. The grayish precipitate was rapidly replaced by a fine black precipitate (mercuric sulfide). The supernatant liquid, containing the *o*-mercaptobenzoate, was separated from the precipitate by centrifugation and acidified to approximately pH 2. The white precipitate of *o*-mercaptobenzoic acid was separated from the supernatant liquid by centrifugation. The *o*-mercaptobenzoic acid was extracted into a small amount of ether. After removal of the ether, the *o*-mercaptobenzoic acid was recrystallized from alcohol–water and dried *in vacuo* over P_2O_5 for 24 hr, mp 165–166°.

Analyses of benzoic acid and *o*-mercaptobenzoic acid for ^{18}O enrichment were performed in a Varian M 66 mass spectrometer as described for Z-glycine. Areas under the curves were determined by use of a Technicon integrator–calculator (Model AAG).

In separate experiments, in nonenriched water, the rate of production of *p*-nitrophenol (measured at 402 mμ on aliquots diluted with pH 8.0 buffer) and the rate of change in *o*-mercaptobenzoic acid concentration (determined as free sulfhydryl concentration with 5,5′-dithiobis(2-nitrobenzoic acid)) were determined. These two determinations permitted us to follow roughly the rate of thiol ester formation (*p*-nitrophenol production) and breakdown (increase in mercaptobenzoic acid) (Schonbaum and Bender, 1960). At pH 7.2, the *o*-mercaptobenzoic acid catalyzed hydrolysis of *p*-nitrophenyl benzoate was three to four times faster than the hydroxide ion catalyzed hydrolysis. As indicated by the rate of decrease followed by the rate of increase in *o*-mercaptobenzoic acid concentration the rate of formation of thiol ester was only a little faster than its rate of breakdown. These results, which are in marked contrast to those reported for the *o*-mercaptobenzoic acid catalyzed hydrolysis of *p*-nitrophenyl acetate (Schonbaum and Bender, 1960), are probably a result of steric interference with formation of the intermediate thiol ester. The half-life of the hydrolysis of *p*-nitrophenyl benzoate at 74° was ~110 min. The reaction did not fit a simple order of reaction partially because of a slow but measurable oxidation of *o*-mercaptobenzoic acid during the reaction.

Results

Papain-Catalyzed Hydrolysis. The ^{18}O enrichment of Z-glycine during the papain-catalyzed hydrolysis of *p*-nitrophenyl Z-glycinate in 10% ^{18}O-enriched water and nonenriched water is shown in Figure 1 and Table I. In the presence of 10% ^{18}O-enriched water the amount of ^{18}O incorporated into Z-glycine was essentially the same as the ^{18}O content of the water in the reaction mixture. When the papain was isolated from the reaction performed in the presence of 10% ^{18}O-enriched water and allowed to react with more substrate in nonenriched water there was no ^{18}O enrichment of the isolated Z-glycine.

o-Mercaptobenzoic Acid Catalyzed Hydrolysis. The ^{18}O enrichment of benzoic acid and *o*-mercaptobenzoic acid during the *o*-mercaptobenzoic acid catalyzed hydrolysis of p-nitrophenyl benzoate is shown in Figure 2 and Table II. When the reaction was carried out in 10% ^{18}O-enriched water the ^{18}O content of benzoic acid was increased to nearly that of the ^{18}O content of the water of the reaction mixture. However, there was no ^{18}O enrichment of the *o*-mercaptobenzoic acid.

Discussion

Kinetic studies have indicated that an ionizable group (or groups) of apparent p*K* of 4.2–4.3 and 3.9 is involved in the acylation and deacylation steps, respectively, of papain-catalyzed hydrolyses (Whitaker and Bender, 1965). From the p*K* and also from the absence of an appreciable temperature effect on the ionization (Stoc-

TABLE I: Mass Spectrometer Analyses of Z-glycine Produced by Enzymatic Hydrolysis of *p*-Nitrophenyl Z-glycinate.

	First Z-glycine[a]			Second Z-glycine[b]		
	Mass			Mass		
Control[c]	209	210	211	209	210	211
Theory (%)	87.07	11.52	1.41	87.07	11.52	1.41
Experimental (%)	88.46	10.27	1.27	88.46	10.27	1.27
Random[d] (%)	81.56	9.47	8.97	86.26	10.02	3.72
Selective[d] (%)						
a.[e]	81.62	9.48	8.90	85.83	9.96	4.21
b.[e]	80.44	9.34	10.22	88.46	10.27	1.27
Exclusive[d] (%)						
a.[e]	80.44	9.34	10.22	87.00	10.10	2.90
b.[e]	80.44	9.34	10.22	87.73	10.18	2.09
No anhydride intermediate[d] (%)	80.44	9.34	10.22	88.46	10.27	1.27
Found (%)	79.68	9.69	10.63	88.27	10.35	1.38

[a] Hydrolysis in presence of 10.00% ^{18}O-enriched water with substrate to active enzyme ratio of 14.0 [b] Enzyme isolated from first reaction and reacted with 5.45 molar excess substrate in nonenriched water. [c] Reactions performed in nonenriched water. [d] Calculated values based on mechanisms proposed in text, the experimentally determined mass percentages and assuming isotope effect of $^{17}/_{19}$ for attacking hydroxide ion species. [e] See text for meaning of these subdivisions.

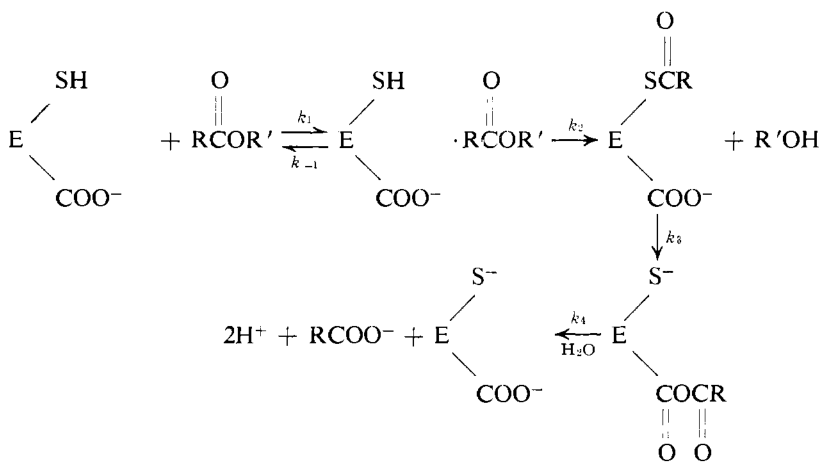
TABLE II: Mass Spectrometric Analyses of Benzoic Acid and *o*-Mercaptobenzoic Acid Produced during Hydrolysis of *p*-Nitrophenyl Benzoate.[a]

	In $H_2{}^{16}O$				In $H_2{}^{18}O$		
	Mass				Mass		
	122	123	124		122	123	124
				Benzoic Acid			
Theory (%)	91.60	7.74	0.66	Symmetrical anhydride, theory[b] (%)	87.40	7.46	5.14
Experimental (%)	91.8	7.56	0.66	No anhydride, theory[b] (%)	83.35	7.04	9.61
				Experimental (%)	83.6	8.55	7.85
	154	155	156		154	155	156
				o-Mercaptobenzoic Acid			
Theory (%)	87.00	8.16	4.84	Symmetrical anhydride, theory[b] (%)	82.90	7.78	9.32
Experimental (%)	87.3	8.10	4.60	No anhydride, theory[b] (%)	87.00	8.16	4.84
				Experimental (%)	87.2	8.00	4.80

[a] Reaction performed for 10 hr at 74° with stoichiometric ratio of *p*-nitrophenyl benzoate and *o*-mercaptobenzoic acid in nonenriched and 10.00% ^{18}O-enriched water. [b] Calculations assumed an isotope effect of $^{17}/_{19}$ for attacking hydroxide ion species.

kell and Smith, 1957), it has been concluded that the group involved is a carboxyl group.

The carboxyl group can function catalytically as a general acid, general base, or as a nucleophile (Bender, 1960a; Fersht and Kirby, 1967a–c). Because of the nature of the activity–pH profile of papain-catalyzed reactions it is not likely to function as a general acid. Equation 1 describes the simplest mechanism one could propose for the papain-catalyzed hydrolysis of *p*-nitrophenyl Z-glycinate if the carboxyl group functions as a nucleophile. The presence of the postulated acyl-enzyme intermediate (thiol ester) has been observed spectro-



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phometrically (Lowe and Williams, 1964; Bender and Brubacher, 1964) and has been isolated (Brubacher and Bender, 1966). Let us consider several possible mechanisms for hydrolysis of the postulated anhydride intermediate (eq 1) in $H_2{}^{18}O$.

1. Random Mechanism. There is equal attack at each of the carbonyl carbons of the anhydride intermediate by hydroxide ions. Under these conditions the carboxyl group of the enzyme will become labeled with ^{18}O. If the labeled enzyme is removed from the reaction and allowed to react with more substrate in the presence of nonenriched water, some of the product will be labeled with ^{18}O.

2. Selective Mechanism. The two carbonyl carbons of the anhydride intermediate are not equally susceptible to attack by hydroxide ions. The two extreme cases are: (a) exclusive attack at the carbonyl carbon contributed by the enzyme, and (b) exclusive attack at the carbonyl carbon contributed by the substrate. In the first case the enzyme would become labeled with ^{18}O while in the second case no ^{18}O would be incorporated into the enzyme. Only the first case would lead to ^{18}O incorporation into the product formed when the enzyme is isolated and reacted with substrate in nonenriched water.

3. Exclusive Mechanism. In the random and selective mechanisms the oxygen atoms of the carboxyl group of the enzyme have been treated as being equivalent. Such may not be the case in an enzyme because of hydrogen bonding, steric factors, etc. In the extreme case then only one of the oxygens may participate in the formation of an anhydride and thus become labeled. During isolation of the labeled enzyme by Sephadex gel filtration (see above) before treatment with more substrate in nonenriched water two possibilities exist: (a) there is randomization of any incorporated ^{18}O between the two carboxyl oxygens, and (b) there is no randomization of ^{18}O between the two carboxyl oxygens during the isolation process. In either case there would be some incorporation of ^{18}O into the product formed during the hydrolysis of substrate in nonenriched water but the absolute amount of incorporation would be different.

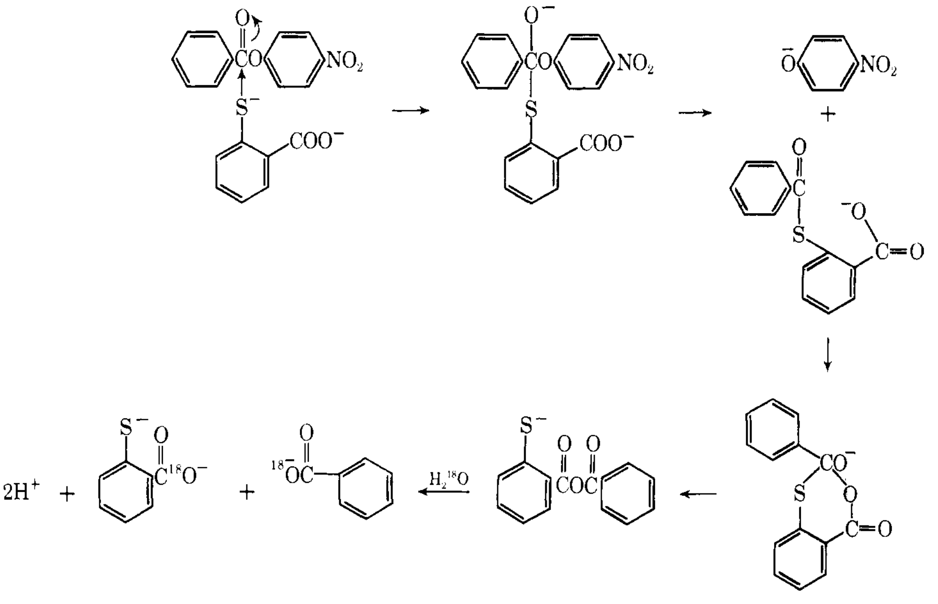
4. No Anhydride Intermediate Involved. In this case the product would be enriched with ^{18}O to the same extent as the ^{18}O content of the water used. There would be no incorporation of ^{18}O into the enzyme or into a product formed after isolation of the enzyme and subsequent reaction with more substrate in nonenriched water.

In Table I are shown the expected results based on the mechanisms postulated above and the results obtained. It will be seen that the Z-glycine produced in the presence of ^{18}O-enriched water is enriched to the extent predicted by the selective mechanism (case b), the exclusive mechanism, and by the no anhydride intermediate mechanism. There was no ^{18}O enrichment of the product when the isolated enzyme was allowed to react with more substrate in nonenriched water. Therefore, the random and exclusive mechanisms are ruled out.

The data do not permit us to distinguish between the selective mechanism (case b) and the no anhydride intermediate mechanism. In the selective mechanism (case b) we have assumed that there is no attack of hydroxide ions at the enzyme carbonyl group. The nature of the postulated anhydride is predicted to be the mixed anhydride of Z-glycine and aspartyl residue 163 of the enzyme. While it is well known that the nature of the alkyl group has a marked influence on the velocity of hydrolysis of carbonyl derivatives (Evans *et al.*, 1938; Bender *et al.*, 1958), we have no way of predicting the influence of the enzyme in permitting selective reaction at only one of the carbonyl groups.

Presumptive evidence for the possibility of an anhydride intermediate in papain-catalyzed hydrolyses is dependent solely upon model systems, particularly the *o*-mercaptobenzoic acid catalyzed hydrolysis of *p*-nitrophenyl acetate (Schonbaum and Bender, 1960). In this reaction convincing evidence is presented that a thiol ester intermediate (thioaspirin) is formed. However, the evidence for an anhydride intermediate is based solely on the close structural analogy between aspirin and thioaspirin. Evidence for an anhydride intermediate in aspirin hydrolysis was supported by a small incorporation (6%) of ^{18}O into salicylic acid from $H_2{}^{18}O$ of the reaction medium (Bender *et al.*, 1958).

Recently, Fersht and Kirby (1967a) have reexamined the hydrolysis of aspirin and have concluded that all data, including absence of ^{18}O incorporation into salicylic acid, rule out a nucleophilic catalysis by the carboxylate group but the data do support general base catalysis. In view of this more recent work on aspirin,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which appeared after the completion of the work with papain reported above, and because of an inability to distinguish between the selective mechanism (case b) and the no anhydride intermediate mechanism, it appeared desirable to determine if there is ^{18}O incorporation into the carboxyl group of *o*-mercaptobenzoic acid from the solvent during its catalysis of ester hydrolysis.

To avoid the problem of differential attack of hydroxide ions at each of the carbonyls of an unsymmetrical anhydride (see above), we used *p*-nitrophenyl benzoate as the ester. Any anhydride formed would be resonably symmetrical and hydrolysis of the anhydride in ^{18}O-enriched water should result in approximately 50% incorporation into the carboxyl groups of both benzoic acid and *o*-mercaptobenzoic acid (eq 2).

The mass spectrometric analysis of benzoic acid (hydrolysis product of *p*-nitrophenyl benzoate) and *o*-mercaptobenzoic acid (catalyst) from hydrolyses performed in 10% ^{18}O-enriched water and in nonenriched water are shown in Table II and Figure 2. Benzoic acid is enriched to the extent of 7.85% in ^{18}O when the reaction is carried out in $H_2{}^{18}O$. The difference between the expected value of 9.61% and the experimental value of 7.85% is most probably due to the participation of Tris in the reaction. In an experiment carried out in an identical fashion but without *o*-mercaptobenzoic acid and for 48 hr (to completion) the ^{18}O enrichment of benzoic acid was 2.55% (three determinations). On the basis of this data and the ratio of $t_{1/2}$ for the reaction in the presence and absence of *o*-mercaptobenzoic acid of 3.74, one calculates an expected ^{18}O enrichment of 7.72%, essentially that found.[2]

Excellent agreement between the theoretical values and the experimental values was found for *o*-mercaptobenzoic acid. *o*-Mercaptobenzoic acid used as a catalyst in 10% ^{18}O-enriched water showed no incorporation of ^{18}O into the carboxyl group. These data unequivocally rule out a nucleophilic attack by the carboxyl group of the thiol ester intermediate (eq 2) to give an anhydride intermediate. If an anhydride intermediate were formed, one might expect more incorporation of ^{18}O into the *o*-mercaptobenzoyl moiety than in the benzoyl moiety of the anhydride because of the electron-withdrawing effects of the *o*-mercapto group. At the very least there should be 50% attack at the carbonyl group contributed by the *o*-mercaptobenzoic acid.

The data do not support the occurrence of an anhydride intermediate in either the papain- or *o*-mercaptobenzoic acid catalyzed hydrolysis of the *p*-nitrophenyl esters used in this study. Therefore, one is led to the conclusion that these hydrolyses involve general base catalysis by the carboxylate group. General acid catalysis at pH 8 is presumably ruled out by the nature of the pH–rate profile. General base catalyzed hydrolysis of the thiol ester intermediate is shown in eq 3 for the papain-catalyzed hydrolysis.

The function of the carboxylate group is to remove a proton from water leaving the hydroxide ion to attack the carbonyl group of the thiol ester. Production of the hydroxide ion within the active site of the enzyme would lead to a marked catalytic effect. The local concentration of an ionized species within the active site of an enzyme (so as to convert an intermolecular reaction into an intramolecular reaction) is equivalent in catalytic capac-

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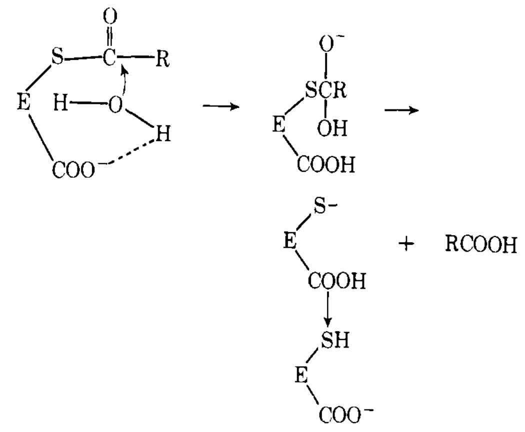
[2] No ^{18}O enrichment of *p*-nitrophenol was found thus ruling out attack at the alkyl group of the ester by water or hydroxide ions. The rate of exchange of oxygen between benzoic acid and water is much too slow to account for the present results (Roberts and Urey, 1939; Bender *et al.*, 1956). Reaction of Tris with phenyl esters is well documented (Jencks and Carriuolo, 1960; Bruice and York, 1961). Under the conditions used here the Tris-catalyzed reaction would be at least twice as fast as the hydroxide ion catalyzed reaction (Bruice and York, 1961). The expected predominant initial reaction products would be *N*-benzoyltris(hydroxymethyl)aminomethane and *p*-nitrophenol (Bruice and York, 1961). To explain the experimental results completely, the breakdown of the amide to give benzoic acid (and other products) must involve participation of the hydroxyl groups of the amide, not hydroxide ions or water. We are presently working on this reaction.



(3)

ity to a 10 M solution of an ion in the bulk medium (intermolecular catalysis) (Bender, 1960a; Koshland, 1962). The removal of a proton as the rate-limiting process in the deacylation step of papain-catalyzed hydrolyses has been indicated from reactions carried out in D_2O (Whitaker and Bender, 1965).

There are a number of examples of carboxyl group catalysis which do involve nucleophilic attack by the carboxylate group. Examples are the intermolecular acetate- or formate-catalyzed hydrolysis of acetic anhydride (Kilpatrick, 1928), propionic anhydride (Kilpatrick, 1930), acetic propionic anhydride (Kilpatrick and Kilpatrick, 1930), and 2,4-dinitrophenyl benzoate (Bender and Neveu, 1958); and the intramolecular carboxyl group catalyzed hydrolysis of 3,5-dinitroaspirin (Fersht and Kirby, 1967b).

Why does the carboxylate group participate as a nucleophile in the intramolecular-catalyzed hydrolysis of 3,5-dinitroaspirin (Fersht and Kirby, 1967b) and as a general base in the intramolecular-catalyzed hydrolysis of aspirin (Fersht and Kirby, 1967a)? Whether the carboxylate group functions as a nucleophile or as a general base in these hydrolyses apparently is determined by the difference in basicity between the potential nucleophile and the leaving group (the phenolic group). Oakenfull *et al.* (1966) have shown that acetate ion does not catalyze the hydrolysis of substituted phenyl acetates by a nucleophilic mechanism if the leaving group is 3–4 p*K* units more basic than the catalyst (acetate ion).

In both the case of the thiol ester intermediate in papain-catalyzed reactions and the thiol ester intermediate (benzoyl-*o*-thiosalicyclic acid) formed in the *o*-mercaptobenzoic acid catalyzed hydrolysis of *p*-nitrophenyl benzoate, the leaving group (sulfhydryl group) has a p*K* of 8.4–8.5 and the carboxyl group has a p*K* near 4 (Whitaker and Bender, 1965; Schonbaum and Bender, 1960). By analogy with the data on the acetate ion catalyzed hydrolysis of substituted phenyl acetates (Oakenfull *et al.*, 1966), one would not expect these hydrolyses to proceed through an anhydride intermediate, an expectation which is in agreement with the experimental data reported here.

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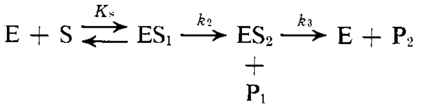
The Presteady-State Kinetics of the Papain-Catalyzed Hydrolysis of Isomeric Nitrophenyl Esters of Carbobenzoxyglycine*

Colin D. Hubbard and Jack F. Kirsch

ABSTRACT: The rate constants for the acylation of papain by the *o*-, *m*-, and *p*-nitrophenyl esters of carbobenzoxyglycine have been determined directly under conditions of $[E] \gg [S]$ by stopped-flow spectrophotometry and the results have been found to agree with the predictions derived from steady-state measurements at $[S] \gg [E]$.

The apparent second-order rate constants did not decrease with increasing enzyme concentrations, implying that the dissociation constant, K_s, is greater than 10^{-4} M for the reactions of the *p*-nitrophenyl ester. The addition of the nucleophile ethanol to the reaction mixture enhanced the over-all rate of reaction by increasing the rate of deacylation but had no effect on the velocity of acylation determined either directly or from the steady-state parameters.

Reactions of nitrophenyl esters with proteolytic enzymes have been particularly instrumental in helping to clarify their mechanism of action. Hartley and Kilby (1952, 1954) made the initial observation that the chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate proceeds in two steps, the first measured by an initial rapid release of *p*-nitrophenol in approximate stoichiometry with the enzyme followed by a slower reaction which is zero order in substrate concentration. The kinetics of both phases were carefully investigated in later work (Kézdy and Bender, 1962; Faller and Sturtevant, 1966, and references therein). Similar experiments have been carried out with trypsin (Stewart and Ouellet, 1959). These initial studies were later extended to steady-state measurements on *p*-nitrophenyl esters of so-called specific substrates, *i.e.*, of *N*-acylamino acids (Bender and Kézdy, 1965, and references therein). The experiments with these compounds, together with other kinds of evidence, have led to the now widely accepted kinetic scheme for the reaction of chymotrypsin and trypsin with esters (eq 1) (Gutfreund and Sturtevant, 1956a,b; Bender and Kézdy, 1965).



$$E + S \underset{}{\overset{K_s}{\rightleftarrows}} ES_1 \xrightarrow{k_2} \underset{\displaystyle P_1}{\underset{+}{ES_2}} \xrightarrow{k_3} E + P_2 \qquad (1)$$

where ES_1 is the noncovalent enzyme–substrate complex, ES_2 an acyl-enzyme with the acyl moiety of the substrate covalently bound to the β-OH of the active site serine residue, P_1 the alcohol released from the substrate, and P_2 the carboxylic acid.

While fewer experiments have been done with the plant sulfhydryl proteases, papain, bromelin, and ficin, the picture that is emerging, at least for papain, the most studied of the three, is that the catalyzed acyl transfer reactions of esters proceed through a very similar pathway with the major chemical difference being that the nucleophilic acyl group acceptor on the enzyme is the β-SH of a cysteine residue (Sanner and Pihl, 1963; Lowe and Williams, 1965a; Brubacher and Bender, 1966). In addition some evidence has been advanced to show that in certain cases the rate of departure of P_1 from the enzyme surface may be of kinetic importance (Henry and Kirsch, 1967, and references therein). Although papain does not react well with *p*-nitrophenyl acetate,[1] it very efficiently catalyzes the hydrolysis of nitrophenyl esters of *N*-acylamino acids (Lowe and Williams, 1965b; Bender and Brubacher, 1966; Kirsch and Igelström, 1966).

The steady-state analysis of eq 1 leads to the following expressions for the Michaelis–Menten parameters, k_{cat} and K_m.

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \qquad (2)$$

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[1] J. F. Kirsch, unpublished experiments.