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Nitrogen Isotope Effects on the Papain-Catalyzed Hydrolysis of *N*-Benzoyl-L-argininamide†

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ABSTRACT: The papain-catalyzed hydrolysis of *N*-benzoyl-L-argininamide shows nitrogen isotope effects (k^{14}/k^{15}) at 25° of 1.021 at pH 8.0, 1.024 at pH 6.0, and 1.023 at pH 4.0. These isotope effects are much larger than those observed in the chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophanamide (O'Leary, M. H., and Kluetz, M. D. (1972), *J. Amer. Chem. Soc. 94*, 3585) and are near the upper limit of nitrogen isotope effects which are observed in reactions in which a bond is broken to an isotopic nitrogen atom. The isotope effects are interpreted in terms of the partitioning of a tetrahedral intermediate. In the case of chymotrypsin, the tetrahedral intermediate partitions rather equally between return to starting materials and loss of ammonia, whereas in the case of papain, the intermediate returns to starting material much more frequently than it decomposes to acyl-enzyme, and carbon–nitrogen bond breaking is entirely rate determining. This interpretation is consistent with the expected properties of the oxygen and sulfur nucleophiles present at the active sites of the two enzymes.

The sulfhydryl-dependent plant protease papain has been studied extensively (Lowe, 1970; Glazer and Smith, 1971). Both the amino acid sequence (Glazer and Smith, 1971) and the X-ray structure of the enzyme (Drenth *et al.*, 1971) have been reported. The minimal mechanism of action is the three-step sequence

$$E + S \rightleftharpoons ES \longrightarrow \underset{+\ P_1}{\text{acyl-E}} \xrightarrow{H_2O} E + P_2 \qquad (1)$$

where ES is the Michaelis complex and acyl-E is the covalent acyl-enzyme intermediate. This acyl-enzyme is formed by the reaction of the substrate with the sulfhydryl group of a cysteine residue at the active site—a reaction which is assisted by the participation of a nearby histidine residue (Husain and Lowe, 1968).

Papain is often compared with chymotrypsin, and it is clear that these two enzymes are similar in many respects. Strong evidence has been presented for the existence of a tetrahedral intermediate in acyl-enzyme formation in the case of chymotrypsin (Fersht and Requena, 1971; Lucas *et al.*, 1973). The presence of such intermediates in nonenzymatic reactions of esters and amides is well documented in many cases (Bender, 1960; Shain and Kirsch, 1968), and it seems natural that such intermediates should also occur in enzymatic reactions. No evidence bearing on the existence of such an intermediate in papain-catalyzed hydrolysis has been presented.

Heavy-atom isotope effects have been used extensively in studies of mechanisms of organic reactions (Fry, 1970), but until recently little use has been made of such isotope effects in studies of enzyme-catalyzed reactions. Heavy atom isotope effects are potentially of great use in studies of enzyme mechanisms because the information they provide is complementary to that provided by rapid-kinetic studies. Whereas the latter provide information about rapid steps in the reaction prior to the rate-determining step, the former provide information about the rate-determining step and any moderately slow steps prior to it.

The pH dependence of nitrogen isotope effects on the chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan-

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amide is suggestive of the occurrence of a tetrahedral intermediate which has a substantial chance of both losing ammonia to form acyl-enzyme and returning to starting material (O'Leary and Kluetz, 1972b). The fate of this intermediate changes with pH. If papain also works by way of a tetrahedral intermediate, then nitrogen isotope effects on the papain-catalyzed hydrolysis of amides might provide information about the fate of this intermediate. In a preliminary communication (O'Leary and Kluetz, 1972a) we reported that the papain-catalyzed hydrolysis of *N*-benzoyl-L-argininamide shows a nitrogen isotope effect substantially larger than that shown by chymotrypsin. The present report extends those studies and compares them with the previous studies of chymotrypsin.

Experimental Section

Materials. Papain (Sigma) was activated by reaction with 0.01 M cysteine in the presence of 10^{-4} M EDTA. *N*-Benzoyl-L-argininamide hydrochloride was purchased from Sigma and was recrystallized several times from acetone–water before use, mp 127° (lit. 127–129° (Whitaker and Bender, 1965)). Norit was thoroughly cleaned by washing before use. Water was purified by means of a Millipore Super Q water purification system and was then distilled from dilute H_2SO_4. Buffers were made up using only potassium salts. 10^{-4} M EDTA and 2.5×10^{-4} M cysteine were present in all buffers.

All solutions and materials to be used for measurement of isotope effects were carefully checked for the presence of spurious ammonia by either the Nessler procedure (Umbreit *et al.*, 1964) or by use of an ammonia electrode (Orion Research).

Methods. The papain-catalyzed hydrolysis of *N*-benzoyl-L-argininamide was followed spectrophotometrically at 280 nm using a Gilford Model 222 photometer attached to a Beckman DU monochromator. The same slit setting was used for all measurements. The reliability of the assay procedure was confirmed by use of the ammonia electrode.

For isotope effect measurements, 100 ml of 0.01 M substrate in buffer containing cysteine and EDTA was equilibrated at 25.0° for at least 30 min, after which a known quantity of activated, desalted papain was added. A 3-ml portion of this solution was removed for uv monitoring of the progress of the reaction, and the remainder of the solution was divided into a 10-ml portion and an 87-ml portion. Additional enzyme was added to the 10-ml portion in order to hasten the completion of amide hydrolysis. When the uv assay indicated that the 87-ml sample had reached 10% hydrolysis (0.5–1 hr), 2.25 ml of 0.1 M $HgCl_2$ solution was added to the 87-ml solution in order to stop the reaction. Unreacted substrate was then removed by extraction with four successive 1-g portions of Norit, and enzyme was removed by ultrafiltration through an Amicon UM-2 membrane.

The small substrate sample was allowed to react for at least ten times as long as the large sample. In order to ensure that the enzyme had not denatured before the reaction was complete, a small sample of the solution was withdrawn and tested for catalytic activity by addition of a small additional amount of substrate and spectrophotometric monitoring. The remaining solution was subjected to the same $HgCl_2$, Norit, and ultrafiltration procedure as was the 10% sample.

The two ammonia solutions resulting from the above procedure were steam distilled, concentrated, oxidized to N_2, and analyzed as previously described (O'Leary and Kluetz, 1972b).

Results

Nitrogen isotope effects on the papain-catalyzed hydrolysis of *N*-benzoyl-L-argininamide have been measured at three pH values and at various ionic strengths. The isotope effects are calculated from measurements of isotopic composition (^{15}N/^{14}N) of two samples of ammonia: a sample isolated after hydrolysis of 10% of the amide present, and a sample isolated from completely hydrolyzed amide (O'Leary *et al.* 1970). The ammonia samples were converted to N_2 by a slight modification of our previous procedure and analyzed by isotope-ratio mass spectrometry (O'Leary and Kluetz, 1972b). Isotope effects are calculated directly from the isotope ratios for the two samples, with the addition of a small extrapolation of the 10% reaction sample to 0% reaction (Bigeleisen and Wolfsberg, 1958). The latter correction increases the isotope effect by about 0.001.

The measured isotope ratios and the calculated isotope effects are given in Table I. The isotope effect at pH 8.0 is in satisfactory agreement with that obtained independently by Kluetz (O'Leary and Kluetz, 1972a). In spite of the presence of a considerable dependence of the steady-state kinetic parameters on pH and salt concentration (Whitaker and Bender 1965), no clear dependence of isotope effect on either of these factors is apparent. The small variations in isotope effect which are observed are only slightly larger than the errors in the measurements, and are probably not real.

The isotope ratios reported in Table I are decade settings on the isotope ratio mass spectrometer which have been corrected to a constant (though arbitrary) value of the isotope ratio for a standard nitrogen sample. These decade settings thus are not absolute isotope abundances, but are instead directly proportional to the isotope abundances; as a result they can be used directly in the calculation of isotope effects.

In order for these isotope effects to be valid, it is necessary that formation of the acyl-enzyme be irreversible; that is, no reaction of the acyl-enzyme with the product ammonia may occur during formation of the 10% reaction sample. That this condition is met was demonstrated by Brubacher and Bender (1966), who showed that the ratio of nucleophilicities of ammonia and water toward *trans*-cinnamoyl-papain is approximately 2600:1 (assuming that only unprotonated ammonia is reactive and that water is 55 M). The sample which is most likely to have problems due to reaction of the acyl-enzyme with ammonia in our experiments is the 10% reaction sample at pH 8. At 10% reaction the accumulated total ammonia concentration is 10^{-3} M, and the free ammonia concentration is about 5×10^{-5} M. From Brubacher and Bender's ratio of nucleophilicities we calculate that the ratio of rates of reaction of the acyl-enzyme with ammonia and water at this point would be 1:400. Thus, not enough acyl-enzyme reacts with ammonia to interfere with the measurement of the isotope effect.

Discussion

Isotope Effects and Enzyme Mechanisms. Heavy-atom isotope effects are measured by a competitive method involving a mixture of isotopic substrates (in this case, amide containing ^{14}N and ^{15}N). In cases where the principal change is the breaking of a bond to an isotopic atom (in this case, breaking of the amide carbon–nitrogen bond) such isotope effects can be interpreted, provided that the bond-breaking step is irreversible under the conditions of the experiment. Under such conditions, the isotope effect reflects relative rates and isotop

TABLE I: Nitrogen Isotope Effects on the Papain-Catalyzed Hydrolysis of *N*-Benzoyl-L-argininamide at 25.0°.

Isotope Ratio[a] × 10^6		
10% Reaction	100% Reaction	k^{14}/k^{15} [b]
	0.10 M Phosphate Buffer, pH 8.00	
9220	9400	1.0206
9224	9390	1.0190
9218	9402	1.0211
9208	9400	1.0221
9230[c]	9415	1.0211
9215[c]	9399	1.0211
	Mean	1.0208 ± 0.0010
0.01 M Phosphate Buffer, pH 8.00, Containing 0.1 M KCl		
9198	9401	1.0233
9206	9396	1.0217
	Mean	1.0225
0.02 M Phosphate Buffer, pH 8.00, Containing 0.25 M KCl		
9195	9396	1.0231
9197	9398	1.0231
	Mean	1.0231
	0.10 M Phosphate Buffer, pH 6.00	
9192	9402	1.0241
9197	9411	1.0246
9195	9398	1.0233
9187	9397	1.0241
	Mean	1.0240 ± 0.0005
0.01 M Phosphate Buffer, pH 6.00, Containing 0.1 M KCl		
9182	9380	1.0228
9203	9403	1.0229
9183	9385	1.0232
	Mean	1.0230 ± 0.0002
0.02 M Phosphate Buffer, pH 6.00, Containing 0.28 M KCl		
9201	9401	1.0229
9208	9402	1.0223
	Mean	1.0226
	0.10 M Acetate Buffer, pH 4.00	
9186	9383	1.0226
9160	9366	1.0237
9174	9377	1.0233
9173	9377	1.0233
	Mean	1.0232 ± 0.0005
0.02 M Acetate Buffer, pH 4.00, Containing 0.3 M KCl		
9215	9406	1.0218
9204	9416	1.0243
	Mean	1.0230

[a] Isotope ratios are mass spectrometer readings for the ratio of peaks *m/e* 29 to 28, corrected to a constant value of the isotope ratio for the nitrogen standard. In addition to the stated phosphate or acetate, all buffers contained 10^{-4} M EDTA and 2.5×10^{-4} M cysteine unless otherwise noted. [b] Corrected to 0% reaction. [c] No cysteine was present in the buffer.

effects on reaction steps up through the bond-breaking step; subsequent steps do not affect the observed results. Thus, in enzyme-catalyzed amide hydrolysis, it does not matter whether acylation or deacylation is rate determining in the overall reaction; the nitrogen isotope effect reflects only the acylation portion of the mechanism.

Unlike hydrogen isotope effects, which may be appreciable for hydrogens which are several atoms removed from the reactive site, heavy-atom isotope effects are short-range effects and are sizable only for reaction steps in which a bond is being made or broken to the isotopic atom. As a result, only the carbon–nitrogen bond-breaking step in amide hydrolysis will show an isotope effect.[1]

If eq 1 is expanded to include the postulated tetrahedral intermediate, then the acylation mechanism for chymotrypsin or papain can be written as

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_2 \xrightarrow{k_3} \text{Acyl-E} + P_1 \qquad (2)$$

where ES_1 is the Michaelis complex and ES_2 is the tetrahedral intermediate. If this equation correctly describes the reaction, then the observed nitrogen isotope effect is given by

$$\text{obsd } \frac{k^{14}}{k^{15}} = \frac{k_3{}^{14}/k_3{}^{15} + R_2(1 + R_1)}{1 + R_2(1 + R_1)} \qquad (3)$$

where $R_1 = k_2/k_{-1}$ and $R_2 = k_3{}^{14}/k_{-2}$ (O'Leary and Baughn, 1972). It has been assumed in the derivation of eq 3 that only k_3 varies with isotopic substitution. This derivation and the following discussion also require that ES_2, the postulated tetrahedral species, be a true *intermediate*, and not merely a transition state.

Thus, nitrogen isotope effects on amide hydrolysis depend on the actual isotope effect on the bond-breaking step and on a series of partitioning factors R which reflect the fates of the various intermediates. Mechanisms analogous to eq 2 but having more intermediates have isotope effect equations similar in form to eq 3 (O'Leary and Kluetz, 1972b).

The relationship between the isotope effect which is observed in amide hydrolysis and the isotope effect on the carbon–nitrogen bond-breaking step is governed by the sizes of the R factors. When $R_2(1 + R_1)$ is small (0.1 or less), then the carbon–nitrogen bond-breaking step is entirely rate determining, and the observed isotope effect is nearly equal to $k_3{}^{14}/k_3{}^{15}$. When $R_2(1 + R_1)$ is near unity, then an isotope effect is observed, but it is smaller than the isotope effect on the bond-breaking step. Such seems to be the case with chymotrypsin (O'Leary and Kluetz, 1972b) and with carbon isotope effects on the enzymatic decarboxylation of acetoacetic acid (O'Leary and Baughn, 1972). Alternatively, if $R_2(1 + R_1)$ is much larger than unity, then carbon–nitrogen bond breaking is no longer rate determining, and the observed isotope effect approaches unity. Carbon isotope effects on the oxidative decarboxylation of isocitric acid are of this type (O'Leary, 1971). In no case can the observed isotope effect be greater than the isotope effect on the carbon–nitrogen bond-breaking step.

In order to use eq 3 for interpreting isotope effects in enzymatic reactions, we would like to predict values of $k_3{}^{14}/k_3{}^{15}$. *Ab initio* calculation of such isotope effects is still impossible

[1] If amide hydrolysis occurs by way of a tetrahedral intermediate, then there might be a small nitrogen isotope effect on the formation of the tetrahedral intermediate because the carbon–nitrogen bond in an amide is appreciably stronger than that in an ordinary amine, whereas that in the tetrahedral intermediate is probably like that in an ordinary amine. However, this effect is probably much smaller than the isotope effect on decomposition of the tetrahedral intermediate because the change in carbon–nitrogen bond order in going from amide to tetrahedral intermediate is probably smaller than the change in going from the tetrahedral intermediate to the transition state in which the carbon–nitrogen bond is broken.

TABLE II: Nitrogen Isotope Effects on Some Organic Reactions.

Reaction	k^{14}/k^{15}	Ref
$C_6H_5CONH_2 + OH^- \rightarrow C_6H_5CO_2^- + NH_3$	1.004	*a*
$p\text{-}NO_2C_6H_4CH_2CH_2N^+(CH_3)_3 + OH^- \rightarrow p\text{-}NO_2C_6H_4CH{=}CH_2 + H_2O + (CH_3)_3N$	1.024	*b*
$C_6H_5CH_2CH_2N^+(CH_3)_3 + OH^- \rightarrow C_6H_5CH{=}CH_2 + H_2O + (CH_3)_3N$	1.0078	*c*
$C_6H_5CH_2ONO_2 + OH^- \rightarrow C_6H_5CHO + H_2O + NO_2^-$	1.0196	*d*
$C_6H_5CH(CH_3)N{=}NCH(CH_3)C_6H_5 \rightarrow N_2$ + other products	1.023	*e*
$p\text{-}NO_2C_6H_4NH_2 + OH^- \rightarrow p\text{-}NO_2C_6H_4O^- + NH_3$	1.000	*f*
$C_6H_5N_2^- + H_2O \rightarrow C_6H_5OH + N_2 + H^+$	1.045	*g*

[a] K. G. Harbison, unpublished results cited in O'Leary and Kluetz (1972b). [b] Hodnett and Sparapany (1964). [c] Smith and Bourns (1970). [d] Buncel and Bourns (1960). [e] Seltzer and Mylonakis (1967). [f] Ayrey and Wylie (1970). [g] Brown and Drury (1965).

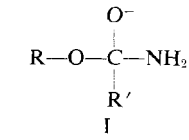
for most molecules of biochemical interest, both because of the difficulty of making adequate vibrational analyses on molecules of such size, and because of a basic lack of knowledge of the amount of bonding to the isotopic atom at the transition state. The best alternative is to predict a range of possible values for k_3^{14}/k_3^{15} by using experimental values of isotope effects obtained in reactions of known mechanism. This approach has been very successful with respect to carbon isotope effects on enzymatic decarboxylations (O'Leary *et al.*, 1970; O'Leary and Baughn, 1972).

A number of nitrogen isotope effects on organic reactions are given in Table II. In reactions where carbon–nitrogen bond breaking is clearly the rate-determining step, the nitrogen isotope effect is ordinarily between 1.010 and 1.025, except in cases where the carbon–nitrogen bond being broken is appreciably stronger than a single bond (decomposition of benzenediazonium ion, for example). The change in isotope effect due to small changes in the structure of the substrate is large, as is the change in isotope effect due to changes in the attacking reagent.

The Papain Mechanism. A large and apparently pH independent nitrogen isotope effect is observed in the papain-catalyzed hydrolysis of *N*-benzoyl-L-argininamide. This isotope effect is considerably larger than the 1.006–1.010 observed in the chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophanamide (O'Leary and Kluetz, 1972a) or in hydroxide induced amide hydrolysis (K. G. Harbison, unpublished results cited in O'Leary and Kluetz (1972b)). In fact, the papain isotope effect is as large as the maximum isotope effects observed in carbon–nitrogen bond-breaking reactions (Table II). This indicates that the carbon–nitrogen bond-breaking step is rate determining in the acylation of papain ($R_2(1 + R_1)$ is considerably less than 1) and may be the only kinetically significant step in the acylation. The absence of a pH dependence of the isotope effect is consistent with this interpretation.

A much more difficult question is whether or not the acylation of papain proceeds by way of a tetrahedral intermediate. There is considerable evidence for the occurrence of such intermediates in a variety of organic reactions (Bender, 1960), in the acylation of chymotrypsin (Fersht and Requena, 1971; Lucas *et al.*, 1973) and in model reactions involving thiol esters (Connors and Bender, 1961; Barnett and Jencks, 1969), but there is no evidence pertaining to such an intermediate for papain. In the following discussion we will show that the nitrogen isotope effects observed in papain-catalyzed hydrolysis are consistent with the occurrence of such an intermediate.

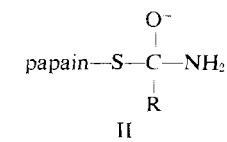
In the case of amide hydrolysis involving an oxygen nucleophile, the tetrahedral intermediate which is formed has the structure



I

where R = chymotrypsin for the enzymatic reaction, and R = H for the reaction with hydroxide. When chymotrypsin reacts with *N*-acetyl-L-tryptophanamide, the partitioning of this intermediate ($R_2(1 + R_1)$) is near unity. This partitioning is also near unity in the reaction of hydroxide ion with benzamide (Bunton *et al.*, 1968). However, when the nitrogen substituent is a better leaving group than NH_3, the partitioning of the intermediate may be different. In the chymotrypsin-catalyzed hydrolysis of substituted *N*-acetyl-L-tryptophananilides, the observed rate is independent of the basicity of the aniline being formed (Philipp *et al.*, 1973). A similar phenomenon is observed in the nonenzymatic reactions of substituted acetanilides with hydroxide ion (Bender and Thomas, 1961). In these cases it is likely that the partitioning of the tetrahedral intermediates favors carbon–nitrogen bond breaking, thus making tetrahedral intermediate formation the rate-determining step.

The tetrahedral intermediate which would be formed during the papain-catalyzed hydrolysis of amides has the structure



II

The principal difference between intermediates I and II is the substitution of sulfur for oxygen. This substitution has a number of predictable effects on reaction mechanism. Sulfur is both a better nucleophile and a better leaving group than oxygen (Wells, 1963). As a result, the partitioning ratio for II should be smaller than that for I, and for a pair of similar substrates carbon–nitrogen bond breaking should be more completely rate determining for papain than it is for chymotrypsin.

This difference between oxygen and sulfur is also seen in the reactions of amines with esters and thiol esters (Connors and Bender, 1961). The tetrahedral intermediate which is formed when amines react with esters (I, R = alkyl) partitions nearly equally between carbon–oxygen bond breaking and carbon–nitrogen bond breaking, whereas the corresponding intermediate in the reaction of a thiol ester decomposes primarily by carbon–sulfur bond breaking.

Extension of this oxygen–sulfur comparison to chymotrypsin and papain must be made carefully because of the possible presence of new factors not present in model reactions. However, the presence of a histidine residue at the active sites of both enzymes encourages us to make this comparison. The partitioning of the tetrahedral intermediate formed in the reaction of chymotrypsin with *N*-acetyl-L-tryptophanamide is near unity. If the principal difference between this reaction and that of papain with *N*-benzoyl-L-argininamide

is the substitution of sulfur for oxygen, then we would predict that in the latter case, carbon–nitrogen bond breaking would be completely rate determining, as is observed.

A comparison of substituent effects on papain- and chymotrypsin-catalyzed anilide hydrolysis further strengthens this picture. In the case of chymotrypsin, the rate-determining step is formation of the tetrahedral intermediate (Phillipp *et al.*, 1973), and no substituent effect is observed. The greater nucleophilicity of sulfur makes carbon–nitrogen bond breaking rate determining for papain, and an appreciable substitutent effect on papain-catalyzed anilide hydrolysis is observed (Lowe and Yuthavong, 1971).

Thus, the nitrogen isotope effects which we have observed in chymotrypsin-catalyzed amide hydrolysis and papain-catalyzed amide hydrolysis can be explained on the assumption that both reactions occur by way of tetrahedral intermediates. If that is true, then the rate-determining step is somewhat different in the two cases, being rather equally divided between formation and decomposition of the tetrahedral intermediate for chymotrypsin, but being decomposition of the intermediate for papain.

Is the Papain-Catalyzed Reaction Concerted? It should be emphasized that the possibility still exists that papain does not operate by way of a tetrahedral intermediate. The difference between nitrogen isotope effects in chymotrypsin-catalyzed amide hydrolysis and papain-catalyzed amide hydrolysis might be due to the occurrence of a tetrahedral intermediate in the former case, but not in the latter. In such a case, the larger isotope effect observed with papain would be due to the lack of a dimunution of the observed isotope effect by the $R_2(1 + R_1)$ in eq 3. Convincing data which would allow us to eliminate this possibility are not available. The lack of a pH dependence in the observed isotope effect is consistent with such a possibility.

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