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Cryoenzymology of Chymotrypsin: The Detection of Intermediates in the Catalysis of a Specific Anilide Substrate†

Anthony L. Fink

ABSTRACT: The reaction between chymotrypsin and *N*-acetyl-L-phenylalanine *p*-nitroanilide has been studied at subzero temperatures in fluid aqueous dimethyl sulfoxide solvent. Following initiation of the reaction at temperatures as low as −90 °C, a series of four reactions prior to the normal rate-limiting step (acylation) was detected spectrophotometrically. Various experimental observations have led to the following interpretation of these reactions. Reaction 1 corresponds to the binding of substrate yielding the initial Michaelis complex. Reactions 2 and 3 are two pH-independent reactions, ascribed to substrate-induced changes in the positions of active-site groups. Reaction 4 is a pH-dependent reaction (pK = 5.9) which involves the imidazole of His-57 but which is not the formation of a tetrahedral intermediate, oxazolinone, or acyl enzyme. The slowest detected step corresponded to the acylation reaction. No evidence for the accumulation of a tetrahedral intermediate was obtained. Spectral, kinetic, and thermodynamic data for these reactions are presented, as is justification for the relevance of these findings to the reaction under physiological conditions. These results demonstrate the utility of subzero temperatures in enzyme mechanism studies, especially with regard to allowing the accumulation of intermediates which may be quite stable at appropriate values of pH and low temperature.

Although chymotrypsin-catalyzed reactions have been very extensively studied, relatively little information is available concerning the dynamic processes occurring during the catalysis. The currently accepted pathway for the reaction involves a covalent acyl enzyme intermediate, EA in (1), in addition to the initial noncovalent Michaelis complex, ES (Bender and Kilheffer, 1973; Fastrez and Fersht, 1973b).

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E + P_2 \qquad (1)$$
$$\qquad\qquad\qquad\quad + \qquad\qquad\quad P_1$$

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For specific amide substrates, the acylation reaction (k_2) is rate determining. The formation of the acyl enzyme involves the "charge-relay" system (Blow et al., 1969) consisting of the catalytic triad Ser-195, His-57, Asp-102 (Hunkapiller et al., 1973; Robillard and Shulman, 1972; Brinigar and Chao, 1975). Several studies of chymotrypsin catalysis have suggested the existence of additional intermediates to those shown in (1): investigations have been done using *N*-furylacryloyl substrate derivatives (Hess et al., 1970; Yu and Viswantha, 1969; Coletti-Previero et al., 1970), nitrogen isotope effects (O'Leary and Kluetz, 1972), and the pH dependence of kinetic parameters (Caplow, 1969; Lucas and Caplow, 1972; Lucas et al., 1973; Fersht, 1972a; Fastrez and Fersht, 1973a; Fersht and Requena, 1971; Fersht and Renard; 1974).

We have been applying the technique of using very low temperatures in conjunction with fluid aqueous organic solvent systems as a means of obtaining mechanistic information about enzyme-catalyzed reactions, for which we suggest the name *cryoenzymology*. This approach is particularly well-suited for the detection and accumulation of intermediates in enzyme-catalyzed reactions since the requisite nonsteady-state, nonturnover conditions may be readily obtained. We have previously shown that 65% aqueous dimethyl sulfoxide and subzero temperatures have no adverse effects on the structure or catalytic activity of chymotrypsin (Fink, 1973a,b; Fink, 1974; Fink and Wildi, 1974).

In the present study *N*-acetyl-L-phenylalanine *p*-nitroanilide was chosen as substrate. A specific amide substrate with a visible chromophore was desired so that changes in the substrate spectrum could be observed in a region where the enzyme was transparent. Since the chromophoric probe is immediately adjacent to the bond to be cleaved in the catalytic reaction, it should act as a sensitive monitor of events affecting the actual bond-breaking step(s), as well as changes in the environment of the leaving group in the active site. By initiating the reaction at a low temperature, we planned to slow the overall catalytic reaction so that acylation would not occur, and the preceding reactions would become slow enough so that they could be readily monitored and their products accumulated. In this report we present evidence for the existence of at least two additional intermediates on the productive catalytic pathway which occur between the Michaelis complex and the acyl enzyme.

Experimental Section

Materials. α-Chymotrypsin, three-times recrystallized, from Worthington or Sigma, was further purified by affinity chromatography using Agarose-ε-aminocaproyl-D-tryptophan methyl ester. For experiments requiring high enzyme concentrations, the affinity chromatography step was omitted. δ-Chymotrypsin, chymotrypsinogen, and diisopropylphosphorylchymotrypsin (Sigma) were used without further purification. The δ enzyme was used at pH values above 7.6. 3-Methylhistidine-57-chymotrypsin was prepared by the method of Henderson (1971) and purified by affinity chromatography. Dimethyl sulfoxide, reagent grade, was distilled from calcium hydride under vacuum at 37 °C. Doubly distilled water and reagent grade buffer materials were used in the preparation of aqueous buffer solutions. *N*-Acetyl-L-phenylalanine *p*-nitroanilide was synthesized using the dicyclohexylcarbodiimide method (Greenstein and Winitz, 1961) and recrystallized from ethyl acetate–hexane, mp 262–264 °C.

Methods. Enzyme activity was determined using *N-trans*-cinnamoylimidazole burst titrations (Schonbaum et al., 1961). Aqueous dimethyl sulfoxide buffer solutions were prepared as follows. Chloroacetate (for pH* 3.5–5.5)[1] or acetate (for pH* 5.5–7.5) buffer, 0.05 M, 35 parts by volume, was cooled to 0 °C and 65 parts by volume dimethyl sulfoxide was added slowly with stirring. The aqueous buffer components were made up to contain sufficient KCl that the final ionic strength of the aqueous organic buffer was 0.1 M. The pH* of the reaction mixtures was measured before and after the experiment using a Radiometer pH meter and electrodes at 25 °C. The pH* value at lower temperatures was estimated from the linear dependence of pH* on temperature (Maurel et al., 1975). For the buffers used, the changes in pH* were quite small, e.g., 0.2 pH* unit for a 50 °C drop in temperature.

The low-temperature spectrophotometer experiments were carried out in the following manner. A special quartz cell consisting of three concentric cylinders was used. The inner chamber held the sample and was surrounded by the coolant jacket. The outer cylinder contained a vacuum to prevent condensation on the optical faces. A Heto Ultra Cryotherm constant-temperature bath, containing ethanol as circulant, was used to maintain the temperature constant to within ±0.15 °C for the duration of the experiment. Sample temperatures were monitored continuously using a thermocouple and digital voltmeter. The sample cell was mounted in a Cary 118 spectrophotometer and pre-cooled to the desired temperature. Enzyme and substrate solutions were prepared by mixing a small aliquot of stock solution in 2 ml of 65% aqueous organic buffer at 0 °C. These solutions were taken up in syringes mounted on a special mixing device consisting of a six-jet tangential mixer constructed of Teflon or stainless steel. The mixer and syringes were immediately cooled to the desired temperature and the solutions mixed and injected into the sample cell in the spectrophotometer. Alternately a small aliquot of enzyme, diluted 1:4 with 65% Me_2SO buffer, was added directly to the precooled substrate–buffer solution in the sample cell. Care was taken to maintain enzyme solutions containing dimethyl sulfoxide below 10 °C to prevent denaturation.[2]

Two methods of monitoring the progress of the reaction were used. In one the wavelength was fixed, and changes in absorbance were recorded as a function of time. Alternately the sample was scanned repeatedly over the range 500 to 300 nm. Since neither enzyme, substrate, nor product absorbs at 500 nm, this wavelength provided a means of monitoring the baseline stability. If changes occurred at 500 nm, the experiment was disregarded. Control experiments with enzyme alone or substrate alone were frequently run and showed no time-dependent spectral changes. Kinetic analysis of experiments in which more than one reaction had similar rates was done using a computerized iteration procedure to evaluate the observed rate constants (Wiberg, 1965). Most kinetic experiments were repeated at least twice.

Results

The Turnover Reaction. The release of *p*-nitroaniline,

[1] pH* is the apparent protonic activity in the aqueous organic solvent (cf. Hui Bon Hoa and Douzou, 1973).

[2] The position of the reversible transition between native and denatured enzyme in 65% dimethyl sulfoxide occurs at ~10 °C at pH* 4.0 (Fink and Grey, unpublished results).

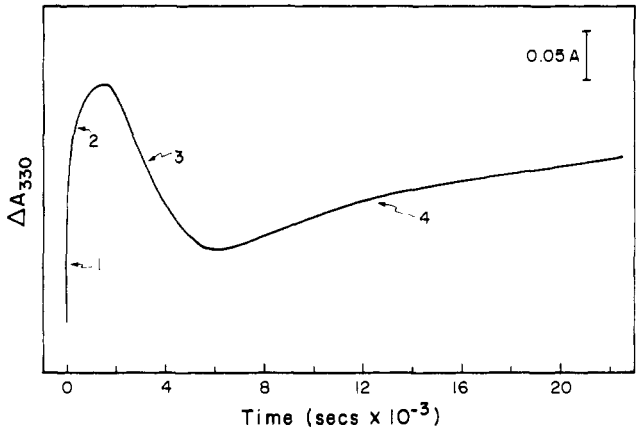


FIGURE 1: Changes in absorbance at 330 nm as a function of time in the reaction between α-chymotrypsin and *N*-acetyl-L-phenylalanine *p*-nitroanilide. The reaction conditions were −56.0 °C, pH 7.6, 65% aqueous dimethyl sulfoxide, $E_0 = 5.0 \times 10^{-5}$ M, $S_0 = 1.1 \times 10^{-5}$ M. The numerals 1–4 correspond to the reactions referred to in the text.

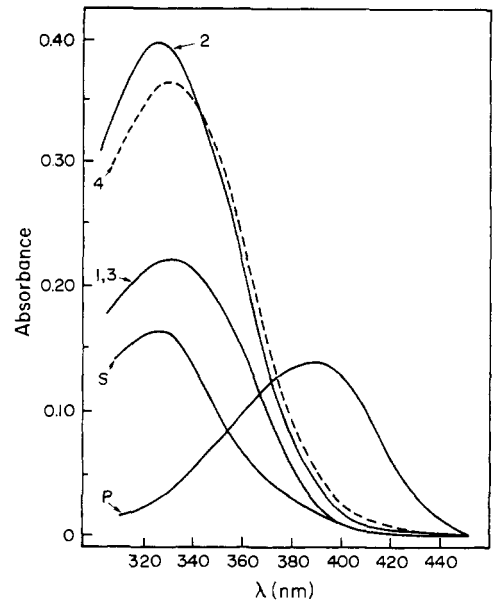


FIGURE 2: Spectra for the products of reactions 1–4 in Figure 1, and those of the substrate and *p*-nitroaniline for comparison. The experimental conditions were the same as those for Figure 1.

corresponding to turnover, was monitored either by repetitive spectral scans, or by fixed wavelength measurements at or above 410 nm, where the substrate has no absorbance. The reaction was followed under pseudo-first-order conditions ($S_0 \ll K_m$) or by the initial velocity technique, and with either excess enzyme or excess substrate. The limited substrate solubility prevented accurate determination of k_{cat} and K_m. Values of k_{cat}/K_m were obtained from pseudo-first-order runs (e.g., $k_{cat}/K_m = 0.5$ M^{-1} s^{-1} at pH* 7.6, 0 °C). The pH and temperature dependence of turnover were determined from initial velocities. From the Arrhenius plot (Figure 3) a value of $E_a = 13.1 \pm 1.5$ kcal/mol was obtained at pH* 7.6. From plots of $1/v$ vs. $[H^+]$, the pK was found to be 7.6 at 0 °C. Excellent agreement between the observed absorbance increases at 410 or 430 nm and those calculated for the substrate concentration employed was obtained in the organic solvent system. Under conditions of $K_m \gg S_0 \gg E_0$ or $K_m \gg E_0 \gg S_0$, the observed first-order rate constants were directly proportional to the concentration of the limiting reagent. Difficulties were encountered in some turnover experiments due to precipitation. Under identical conditions, enzyme, or substrate alone, would not precipitate.

Nonturnover Reactions. The reaction between enzyme and substrate was observed under nonturnover conditions by using subzero temperatures. The reaction was usually initiated by mixing enzyme and substrate at temperatures in the −80 to −40 °C region, the progress of the reaction being followed either at fixed wavelength, usually 330 or 350 nm, or by repetitive spectral scans. These experiments were carried out at several pH values over the range pH* 3.5 to 7.6, normally with $E_0 \gg S_0$. A typical plot of the time dependence of the observed absorbance changes is shown in Figure 1. If either enzyme or substrate was omitted from the reaction mixture, no changes occurred. Under conditions of $E_0 \gg S_0$ when the substrate concentration was doubled, the magnitude of the absorbance changes doubled and, when the enzyme concentration was changed, no changes in the intensity of the spectra occurred. As shown in Figure 1, a series of four reactions is observed, prior to turnover. The turnover reaction, as monitored by the release of *p*-nitroaniline, was much too slow to be observed on a reasonable time scale under these conditions. The spectra of the products of these reactions, as well as those of substrate and *p*-nitroaniline, are shown in Figure 2. Similar spectral changes were observed in experiments in which $S_0 \gg E_0$. The reaction was also performed under similar conditions using chymotrypsinogen, diisopropylphosphorylchymotrypsin, and α-chymotrypsin in which the imidazole of His-57 was methylated (Henderson, 1971). With the former two no absorbance changes were noted. With the latter, reactions 2 and 3 were unchanged but reaction 4 was not apparent, and the amplitude of reaction 1 was decreased. Attempts to slow reaction 1 down sufficiently to follow its kinetics by nonstopped-flow techniques were unsuccessful. At −90 °C the reaction was over in a few seconds. Reactions 2 and 3 were pH independent over the range 4.2 to 7.8. Typical values[3] of k_{obsd} were 2.6×10^{-3} s^{-1} for reaction 2 at −55.5 °C ($E_0 = 5.0 \times 10^{-5}$ M, $S_0 = 1.1 \times 10^{-5}$ M) and 2.5×10^{-3} s^{-1} for reaction 3 at −45.0 °C ($E_0 = 2.5 \times 10^{-5}$ M, $S_0 = 1.0 \times 10^{-4}$ M). Reaction 4 showed increasing rates with increasing pH* and had a p$K = 5.9 \pm 0.2$ at 0 °C under conditions of both excess enzyme and excess substrate. For $S_0 \gg E_0$ a linear relationship between increases in substrate concentration and k_{obsd} was noted. Typical[3] limiting values of k_{obsd} were 5.9×10^{-2} s^{-1} at 1.0 °C ($E_0 = 5.4 \times 10^{-5}$ M, $S_0 = 1.5 \times 10^{-4}$ M), and 4.5×10^{-2} s^{-1} at −2.0 °C ($E_0 = 1.3 \times 10^{-4}$ M, $S_0 = 1.5 \times 10^{-5}$ M). Arrhenius plots for reactions 2–4 are shown in Figure 3. From these plots the following activation parameters were calculated. (Values of $\Delta H^{\ddagger}$ are in kcal mol^{-1} and for $\Delta S^{\ddagger}$ in eu.) For reactions 2 and 3 at −55.5 °C, $\Delta H^{\ddagger}$ was 7.8 ± 2.0 and 8.5 ± 2.2, and $\Delta S^{\ddagger}$ was −37 and −32, respectively. At −20.0 °C $\Delta H^{\ddagger}$ was 11.4 ± 2.5 and $\Delta S^{\ddagger}$ was −26 for reaction 4. For reaction 5 $\Delta H^{\ddagger}$ was 13.1 ± 1.5 and $\Delta S^{\ddagger}$ was −2.1 at 0 °C. Reaction 4 was found to be readily reversible. For example, if reactions 1–3 were carried out at −65 °C and the temperature then was raised to −30 °C for reaction 4 and then returned to −65 °C at the completion of reaction 4, the absorbance spectrum showed changes corresponding to the transformation of the product of reaction 4

[3] Representative kinetic data for reactions 2–4 are available from the author on request.

to that of reaction 3. The estimated rates for reactions 2, 3, and 4, calculated for 25 °C, 0% dimethyl sulfoxide, pH 7.5, $E_0 = 5.0 \times 10^{-5}$ M and $S_0 = 1.0 \times 10^{-3}$ M are 2.0×10^3, 9×10^2, 1.4×10^3, and 5.6×10^{-2} s^{-1}, respectively. The accuracy of these values is estimated to be ±50%.

Discussion

The results of this study show that the rate of the turnover reaction in the chymotrypsin-catalyzed hydrolysis of the specific amide substrate *N*-acetyl-L-phenylalanine *p*-nitroanilide can be effectively reduced to zero at subzero temperatures. At suitably low temperatures, a series of time-dependent spectral changes in the *p*-nitroaniline chromophore can be detected which reflect events at the active site. These observations raise several important questions, including the following. Do the observed spectral changes correspond to the transformation of enzyme-substrate intermediates? Are the presumed intermediates on the productive catalytic pathway? What can we infer about the nature of the enzyme-substrate interactions and the structure of the intermediates? What is the relevance of these observations to the reaction under "normal" conditions (no organic solvent, ambient temperatures)?

Clearly, if mechanistically significant information is to be obtained from studies at subzero temperatures, it is desirable that the reaction at such low temperatures be shown to be analogous to that under normal conditions. In some cases this can be done by comparison with data obtained from rapid reaction techniques (Douzou et al., 1970). It is our contention that the results of the present investigation at subzero temperatures are pertinent to the "normal" catalytic reaction. From previous studies with other substrates of chymotrypsin, we have shown that 65% aqueous dimethyl sulfoxide and subzero temperatures do not significantly perturb the overall catalytic pathway. For example, the magnitude of the rate constant, the pH dependence, and the energy of activation for the acylation and deacylation reactions of several ester substrates are entirely in accord with those expected based on the values at +25 °C, 0% dimethyl sulfoxide, (Fink, 1973a,b; Fink, 1974; Fink and Wildi, 1974; A. L. Fink and G. McGarraugh, unpublished observations). In fact we have also been able to isolate acylchymotrypsins using gel filtration at subzero temperatures (Fink, 1973b).

For the reaction with *N*-acetyl-L-phenylalanine *p*-nitroanilide, we can conclude that any intermediates formed between enzyme and substrate are either on the productive catalytic pathway or in readily reversible equilibrium with such a pathway since raising the temperature sufficiently results in turnover (i.e., there can be no irreversibly formed, dead-end complexes). Since the observed spectral changes (Figure 1) are absent when no enzyme is present or when chymotrypsinogen or diisopropylphosphorylchymotrypsin is used, we can be confident that the observed changes involve substrate bound in the active site.

All the available data are consistent with reaction 1 being the binding of substrate. For example, when the active site is blocked either by the diisopropylphosphoryl group or in the zymogen, no binding would be expected. For the simple binding reaction (2)

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \qquad (2)$$

the observed rate, under conditions of excess enzyme, is given by $k_{obsd} = k_1 E_0 + k_{-1}$. Reasonable values for k_1 and k_{-1} at 25 °C are 1.5×10^7 M^{-1} s^{-1} and 4×10^{-3} s^{-1}, respectively (Philip and Bender, 1973). Although values of $\Delta H^{\ddagger}$ for k_1 and k_{-1} are not available, they will not be more than 5 and 8 kcal/mol, respectively (Fastrez and Fersht, 1973b; Bender et al., 1964). Using these values, we estimate a value of $k_{obsd} \geq 0.1$ s^{-1} at −100 °C under the experimental conditions. Thus the observation that reaction 1 is complete within a few seconds at −90 °C is in accord with expectations. A complication with anilide substrates is that of nonproductive binding (Fastrez and Fersht, 1973b). In fact it has been proposed that the predominant contribution to K_m for most anilide substrates would be the nonproductive binding mode, i.e., $K_s > K_{NP}$ where K_{NP} is the dissociation constant for the nonproductive binding complex (ES_{NP}) (Fastrez and Fersht, 1973b). Making the reasonable assumption that the rates for formation and collapse of ES_{NP} would be within a couple of orders of magnitude of those for the productive complex, we conclude that reaction 1 also represents the binding of S in the nonproductive mode (i.e., the nitroaniline moiety in the hydrophobic pocket). Other possible reactions which are of concern as far as being responsible for reaction 1 are those of dissociation of enzyme dimers and of conformational isomerism involving the Ile-16–Asp-194 salt bridge (Fersht, 1972b). At the concentrations of chymotrypsin used in these experiments one would not expect any significant amount of dimer to be present (Pandit and Narasinga Rao, 1974). Furthermore calculations on the kinetics of the dedimerization indicate that it would be much faster than the rate of reaction 2 (M. Gilleland, personal communication). The temperature dependence of the equilibrium between active (E_a) and inactive (E_i) conformers is such that lower temperatures favor the E_i form (Fersht, 1972b). However, our experimental procedures are such that the temperature of the enzyme solution is dropped very rapidly. At the low temperatures at which the experiments were run, the rate of transformation of E_a into E_i is essentially zero. We have been able to observe small changes in the uv absorption of the enzyme (in the absence of substrate) at subzero temperatures which we be-

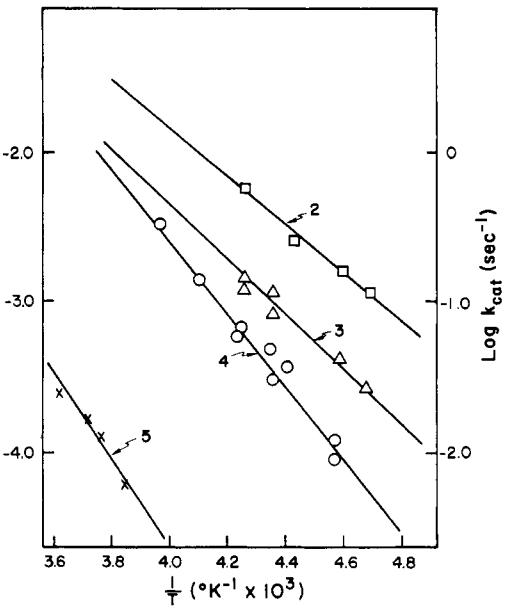
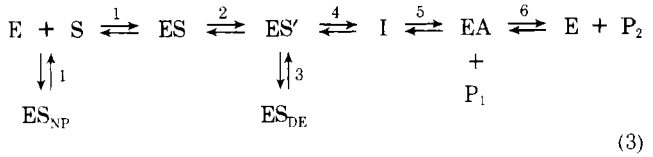


FIGURE 3: Arrhenius plots for reactions 2–5. For reactions 2–4, the conditions were $E_0 = 5.0 \times 10^{-5}$ M, $S_0 = 1.1 \times 10^{-5}$ M. For reaction 5 (turnover), the rate constant used was k_{cat}, $E_0 = 1.0 \times 10^{-4}$, $S_0 = 2.0 \times 10^{-3}$ M. All reactions were in 65% aqueous dimethyl sulfoxide, pH 7.6.

lieve correspond to the conversion of E_a to E_i (A. L. Fink and N. Good, unpublished results). The rates of the observed reaction, which fit on an Arrhenius plot with those of Fersht (1972b), become extremely slow below −30 °C and are much slower than any of the reported catalytic steps. Burst titration experiments using the *p*-nitrophenyl esters of *N*-acetyl-L-tryptophan and *N*-acetyl-DL-phenylalanine at subzero temperatures have shown that, under the conditions used in the present experiments, the active site normality is in good agreement with that obtained from cinnamoylimidazole titrations at pH 5.0, 25 °C, 0% dimethyl sulfoxide (A. L. Fink, unpublished results). The active form of the enzyme is thus "trapped" by a combination of the very slow rate of transformation at low temperatures and by the presence of the substrate.

Reactions 2 and 3 are very similar in several aspects. Since the spectra of the products of these reactions indicate no *p*-nitroaniline, they cannot involve the formation of an oxazolinone or acyl enzyme. The presence of reactions 2 and 3 with 3-methylhistidine-57-chymotrypsin, as well as their pH independence, indicates that they do not involve the catalytic function of the imidazole of His-57 and the charge-relay system. The most reasonable interpretation of these reactions is that reaction 2 is a substrate-induced conformational change (Koshland, 1958), and reaction 3 a subsequent additional conformational change. By conformational change in this context we mean small changes in the positions of some of the atoms (enzyme or substrate or both) in the active site vicinity, not major changes in the position of the peptide backbone. Circular dichroism studies with the corresponding methyl ester substrate indicate that no substantial conformational changes occur in the enzyme during this stage of the catalytic reaction (A. L. Fink and K. Angelides, unpublished results). It is generally accepted that binding of substrate may induce conformational responses in enzymes which result in the proper orientation of the catalytic groups with respect to the substrate (Citri, 1973). The question arises as to whether both reactions 2 and 3 are on the productive catalytic pathway. We have observed reactions analogous to 2 and 3 in the chymotrypsin-catalyzed hydrolysis of other substrates, including esters (Fink et al., 1975). Since these reactions have always been faster than the acylation (at least with ethyl and methyl esters) (Fink et al., 1975), their rates are consistent with their being the formation of productive complexes. At present there is insufficient evidence to rule out the possibility that either reaction 2 or 3 represents the formation of a readily reversible dead-end complex (ES_{DE}), e.g., (3).



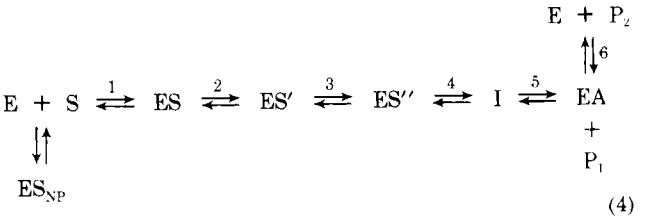
(3)

One means of determining whether reaction 3 involves formation of a dead-end complex would involve finding conditions of pH and temperature where reaction 4 would be expected to be faster than reaction 3 (cf. Figure 3). If under such conditions reaction 3 was not rate limiting with respect to reaction 4, then a scheme of the sort shown in (3) would be implicated.

The pH dependence of reaction 4 and its absence with methylchymotrypsin indicate the involvement of His-57 as a catalytic agent. The p*K* for reaction 4 is perturbed downward from the intrinsic p*K* of 6.8 of the free enzyme (Fersht and Renard, 1974). Perturbations of the kinetically determined p*K* in the chymotrypsin-catalyzed hydrolysis of amides to values around 6.1 have been previously reported (Lucas et al., 1973; Fersht and Requena, 1971) and have been shown to be due to changes in the p*K* on substrate binding (Fersht and Renard, 1974). Since the spectrum of the product of reaction 4 shows no evidence of any *p*-nitroaniline, the possibility that reaction 4 involves the formation of an oxazolinone or a tight acyl enzyme-*p*-nitroaniline complex can be ruled out. The involvement of the charge-relay system suggests the possibility that this reaction could be the formation of a tetrahedral intermediate. However, two observations mitigate against such an interpretation. First, the spectrum of such an intermediate would be expected to be similar to that of *p*-nitroaniline (Robinson, 1970); and secondly, the anticipated pH dependence of the collapse of the tetrahedral intermediate to form acyl enzyme is not in accord with that observed for reaction 5. At present the most reasonable interpretation of reaction 4 is that it involves a rearrangement of the positions of the catalytic groups in the charge-relay system and the amide end of the substrate to appropriately orient them for the acylation reaction. Support for such an interpretation is found in a recent report on the properties of the imidazole of His-57 in chymotrypsin and indolylchymotrypsin (Cruickshank and Kaplan, 1975) and from studies of models based on crystallographic studies of the enzyme and enzyme-trypsin-inhibitor complex, both of which indicate the need for movements of the hydroxymethyl group of Ser-195 and of the imidazole of His-57.

Reaction 5, which involves the formation of *p*-nitroaniline, clearly corresponds to the formation of the acyl enzyme. If this reaction were the collapse of a tetrahedral intermediate formed in reaction 4, we would expect the His-57-Asp-102 system to act as an acid catalyst, and thus we would anticipate the rate to increase with decreasing pH. In fact, the opposite of this is observed. The reason for the relatively high p*K* for reaction 5 is not apparent at this time, although it is possible that it may be due to a solvent effect.

The spectral changes observed in the reaction of chymotrypsin with *N*-acetyl-L-phenylalanine *p*-nitroanilide are best interpreted in terms of scheme 3 or 4.



(4)

Reaction 6 may involve several intermediates, possibly analogous to ES, ES′, ES″, and I in which the *p*-nitroaniline is replaced by water.

The kinetic analysis of the data is somewhat complicated by the similar rates for reactions 2 and 3, and reaction 4 at high pH. The estimated rate constants for reactions 2–4, when adjusted to 25 °C and 0% dimethyl sulfoxide, are quite large (see Results). In fact it is not at all surprising that reactions 1–3 are not observable using stopped-flow techniques under normal conditions. The rate reductions obtained for reactions 2–4 at subzero temperatures in this investigation vividly demonstrate the potential of such low-temperature studies. The general form of the observed first-order rate constant in terms of the underlying microscopic constants for a scheme such as (4) is given by $k^{i}_{obsd} = k_{-i}$

$+ k_i/A$, where $A = 1 + K_3 + K_2K_3 (1 + K_1/E_0)$ for $i = 4$, $E_0 \gg S_0$, and $K_1 = k_{-1}/k_1$, $K_2 = k_{-2}/k_2$ etc. (A. L. Fink, submitted). A detailed kinetic analysis in which the underlying microscopic rate constants are obtained from the observed rates will be reported later.

One means of determining whether schemes 3 and 4 are reasonable is to compare them with existing data obtained under "normal" conditions, and to examine the detailed nature of the intermediates formed in reactions 2–4. Original studies which suggested intermediates additional to those shown in (1) in the chymotrypsin-catalyzed hydrolysis of *N*-(2-furyl)acryloyl-L-tyrosine ethyl ester (Barman and Gutfreund, 1966) were subsequently shown to be consistent with (1) (Himoe et al., 1969). An intermediate between ES and EA in the reaction with *N*-(2-furyl)acryloyl-L-tyrosine methyl ester at low pH has been observed spectrophotometrically (Yu and Viswantha, 1969). The rate of formation of this intermediate is similar to that for oxazolinone formation in the corresponding tryptophan substrate (Coletti-Previero et al., 1970) and of the same magnitude as that estimated for reaction 4 of *N*-acetyl-L-phenylalanine *p*-nitroanilide under similar conditions. Using a combination of relaxation and stopped-flow techniques, Hess (Hess et al., 1970) demonstrated the existence of an intermediate between ES and EA using *N*-(2-furyl)acryloyl-L-tryptophan amide as substrate. The rate of formation of this intermediate was pH dependent and P_1 was not released in the reaction. The rate of formation of this compound would be of the same order of magnitude as reaction 4 in the present study. It would seem reasonable to equate the product of reaction 4 (I in (3) or (4)) with the intermediate observed by Hess and co-workers (1970) on the basis of their pH dependence, similar rates, and breakdown to acyl enzyme.

From the varying pH dependence of the ^{15}N kinetic isotope effect in the chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophanamide, O'Leary and Kluetz (1972) have concluded that there must be an intermediate prior to the acyl enzyme in addition to the one observed by Hess and co-workers. Such a compound would be ES′ in (3), for example. Considerable controversy has existed as to whether a tetrahedral intermediate accumulates prior to collapse into the acyl enzyme. The present study shows no evidence for the accumulation of a tetrahedral intermediate, in accord with the observations of Fersht (Fersht and Renard, 1974). Sykes (1969) has shown that binding of *N*-trifluoroacetyl-D-phenylalanine to chymotrypsin apparently induces a conformational change with a rate of the same order of magnitude as reactions 2 and 3.

The fact that conventional techniques have provided evidence for intermediates consistent with those observed at subzero temperatures is strong support for the validity of scheme 3 or 4 being representative of the catalysis under "normal" conditions. Since we have observed reactions analogous to 2 and 3 with ester substrates (Fink et al., 1975; Fink and McGarraugh, submitted), reactions 2 and 3 are probably common to all chymotrypsin-catalyzed reactions.

Relatively little structural information about the nature of the enzyme-substrate interactions can be obtained from examination of the spectra of the products of reactions 1–4. The procedure best suited to yield structural information about the intermediates is x-ray crystallography. Efforts are currently underway to obtain the products of reactions 3 and 4 in crystalline form suitable for diffraction studies at subzero temperatures (Petsko, 1975). Hopefully the x-ray diffraction studies will allow us to do more than just speculate on the role of the intermediates between ES and EA.

Acknowledgment

The technical assistance of Helene Rock, Michael Parrish, and Geoffrey McGarraugh is gratefully acknowledged.

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The Esterification of Dolichol by Rat Liver Microsomes†

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ABSTRACT: The incubation of 1-[^{3}H]dolichols with cell-free preparations from various rat tissues resulted in the formation of a labeled material which possessed the characteristics of synthetic dolichol palmitate. Rat liver microsomes were found to be a good source of the acyltransferase activity, and the properties of the reaction were investigated using microsomal preparations. The reaction did not require ATP, CoA, or Mg^{2+} and was stimulated by the addition of phosphatidylcholine. The esterification of dolichol appears to be similar to the esterification of retinol. The fact that the esterification of dolichol is not depressed even in the presence of a several-fold excess of retinol is evidence that the two reactions are catalyzed by different enzymes.

Dolichols are polyisoprenoid alcohols containing 16 to 22 isoprene units and possessing a saturated α residue. These compounds function in eucaryotes as intermediates in transglycosylation reactions (see review by Hemming, 1974). The richest practical source of dolichol yet discovered is pig liver which contains approximately 100 μg/g of wet weight. More than half of the dolichol in pig liver is present as the fatty acid ester, which occurs in greatest concentration in the nuclear fraction of the cell (Butterworth and Hemming, 1968). Whether the esters represent a storage form of dolichol or serve some other function is not known at this time. In the course of cell culture studies in which [^{3}H]dolichol metabolism was being investigated, we observed the production of what we assumed to be dolichol esters. Since no data are available on the manner in which dolichol esters are formed, we undertook to study this reaction.

The present paper deals with the partial characterization of the reaction product and an investigation of the properties of the enzymatic system. The results indicate that dolichol is esterified by rat liver microsomes in a reaction in which the acyl moiety may be derived from phosphatidylcholine.

Experimental Section

Materials. Pig liver dolichols, a mixture of isoprenologues containing polyprenols with 16 to 22 isoprene units, was subjected to oxidation with the chromium trioxide–pyridine complex followed by reduction with [^{3}H]$NaBH_4$ as recently described (Keenan and Kruczek, 1975) to yield 1-[^{3}H]dolichol. Reversed-phase chromatography and autoradiography revealed a series of labeled polyisoprenoids as the only significantly labeled products. The specific activity of the dolichol used for these experiments (132 μCi/μmol) was calculated by using the molecular weight of the 95-carbon polyprenol, the predominant compound in the mixture as an average molecular weight. The palmityl ester of dolichol was synthesized from [^{3}H]dolichol and palmityl chloride by the procedure of Pinter et al. (1964) and purified by alumina chromatography. Retinol, retinol esters, phytol, and sphingomyelin were obtained from Sigma Chemical Company. Phosphatidylcholine was isolated from egg yolk lipids by chromatography on alumina as described by Luthra and Shetawy (1972). Lysophosphatidylcholine was prepared by the enzymatic hydrolysis of phosphatidylcholine and was also isolated by column chromatography on alumina. Phosphatidylethanolamine was prepared by alumina chromatography of rat liver lipids. Aqueous dispersions of the phospholipids produced by sonication were added to the incubation mixtures.

Enzyme Preparations. Livers obtained from 160–180-g female Sprague-Dawley rats were homogenized with a Potter-Elvehjem type homogenizer in 3 volumes of isotonic sucrose containing 0.001 M EDTA. Nuclei and cell debris were removed by centrifuging at 600*g* for 10 min, and the supernatant was centrifuged 20 min at 22 000*g* to obtain the mitochondrial fraction. The supernatant from this run was spun at 100 000*g* for 1 h to yield a microsomal pellet. Mitochondrial and microsomal pellets were generally suspended in sufficient sucrose solution to give a concentration of 50–60 mg of protein per ml.

For the experiments in which the effect of phosphatidylcholine was demonstrated, a post-mitochondrial supernatant was fractionated on a discontinuous sucrose density gradient as described by Palamarczyk and Hemming (1975). We obtained two fractions, a pellet (fraction I), and

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