Kinetic evidence for an intermediate in the deacylation of monoacetyl-chymotrypsin

(enzyme kinetics/acetyl transfer/active intermediate)

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ABSTRACT Mono[14C]acetyl-chymotrypsin was prepared by treating α-chymotrypsin with a 10-fold molar excess of p-nitrophenyl[14C]acetate at pH 5, and the acylated enzyme was isolated free of excess reagents by gel filtration. Deacylation at pH 6.0 was followed by observing the decrease in acid-precipitable radioactivity and provided a first-order rate constant of 0.02 ± 0.005 min⁻¹. Reactivation of the acetylated protein was followed by continuously monitoring the appearance of esterolytic activity towards α-N-acetyltyrosine ethyl ester. Reactivation at pH 6.0 occurred exponentially with a first-order rate constant of 0.2 ± 0.015 min⁻¹, the reactivated enzyme exhibiting an apparent catalytic constant (kcat) of 1200 ± 60 min⁻¹, which decreased to a value of 945 ± 15 min⁻¹ by an apparent first-order process with a rate constant of 0.025 ± 0.006 min⁻¹. These results are interpreted in terms of a two-step deacylation of monoacetyl-chymotrypsin involving an acetylated intermediate with esterase activity.

The mechanism of chymotrypsin catalyzed hydrolysis of amide and ester substrates has been shown to proceed via an acyl enzyme intermediate (1). Studies with chromophoric imidazoles have provided conclusive evidence for the reactivation of the acyl enzyme being dependent on the deacylation process (2, 3). The reaction of p-nitrophenyl acetate with chymotrypsin at neutral pH has also been shown to involve an acetyl enzyme intermediate (4), and the isolation of the monoacetyl enzyme has been achieved by performing the reaction at pH 5.0 (5). However, studies with [14C]labeled monoacetyl-chymotrypsin (isolated after reaction of chymotrypsin with p-nitrophenyl [14C]acetate) have revealed that its reactivation occurs far more rapidly at pH 8.0 than its deacylation measured under identical conditions (6). In this report, we describe the kinetics of deacylation of monoacetyl-chymotrypsin.

MATERIALS AND METHODS

[14C]NphOAc was prepared by treating a solution of twice crystallized p-nitrophenol (60 μmol) in dichloromethane with [1-14C]acetanhydride (12 μmol, 8.3 mCi/μmol) in the presence of dicyclohexylcarbodiimide (12 μmol) for 2 hr at 25°C. After removal of the dicyclohexyl urea by filtration, the solution was taken to dryness, the residue combined with 100 mg of cold NphOAc, and the mixture recrystallized from chloroform (melting point 74-76°C). The concentration of a stock [14C]NphOAc solution was determined spectrophotometrically by measuring A400 nm formed upon treatment of aliquots of the solution with dilute base (pH 10.0). An ε_M value of 18,300 M⁻¹ cm⁻¹ for the p-nitrophenol liberated was used (7). Specific activity of the NphOAc preparation was 7.89 μCi/μmol.

Abbreviations: NphOAc, p-nitrophenyl acetate; AcTyrEt, α-N-acetyl-L-tyrosine ethyl ester.

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†† Monoaicylchymotrypsin. α-Chymotrypsin (100 mg, 10 μg/ml), in 100 mM KCl adjusted to pH 3.0 was treated with NphOAc (7.5 mg/ml in acetonitrile) to provide a 10-fold molar excess of the reagent with respect to the protein. After readjustment of the mixture to pH 5.0, the reaction was allowed to proceed for 10 min, and was terminated by adjusting the pH to 3.0. After the removal of precipitated reagents (NphOAc and p-nitrophenol) by filtration, the acylated protein was isolated by chromatography over a 2.5 X 60 cm column of Sephadex G-25 (fine) equilibrated with 100 mM KCl at pH 3.0, and the same solution was used as the eluent. Protein fractions were pooled and stored at 4°C. Analysis of the isolated protein for incorporation revealed the presence of (1.01 ± 0.005) mol of the [14C]acyl moiety per mol of chymotrypsin. The concentration of protein was determined by the spectrophotometric procedure, by using an ε_M²900 value of 20.0 (8), and also by the procedure of Lowry et al. (9).

Deacylation of the enzyme was followed by observing the decrease in acid-precipitable radioactivity. In a typical experiment, 8 ml of the acylated protein (6 mg/ml in 100 mM KCl at pH 3.0) was brought to pH 6.0 by the addition of 2 ml of 500 mM phosphate buffer at pH 6.3. The resulting solution was subjected to constant stirring at 25°C. Aliquots (0.5 ml) were removed at desired intervals, mixed with an equal volume of bovine serum albumin (10 mg/ml, 100 mM KCl at pH 3.0) and immediately treated with an equal volume of a trichloroacetic acid solution (15% in water). After 30 min of standing, the precipitated protein was collected by filtration on membrane filters (0.45 μM, Millipore Corp). After repeated washing of the precipitate with cold trichloroacetic acid (5%), the filter was transferred to a vial containing a liquid scintillation cocktail (Aquasol, New England Nuclear) for the determination of residual acid-insoluble radioactivity.

The rate of reactivation of the acetyl enzyme was measured by recording the rate of N-acetyl-L-tyrosine ethyl ester (AcTyrEt) hydrolysis over an extended period of time. In a typical experiment, an aliquot (5-20 μl) of the acetylated enzyme (6 mg/ml) was added to 5 ml of an AcTyrEt solution [10 mM, 0.09 M KCl, 0.10% (vol/vol) isopropanol at pH 6.0], and the rate of esterolytic activity monitored by recording the uptake of NaOH (78.6 mM) in a pH stat (10). The experiment was terminated when the rate of base uptake reached a constant value.

RESULTS AND DISCUSSION

The first-order plot for the deacylation of monoacetyl-chymotrypsin is shown in Fig. 1. Deacylation is slow during the initial 5 min of exposure of the protein to pH 6.0, but occurs exponentially after that time interval. These studies yielded a first-order deacylation rate constant of (0.02 ± 0.008) min⁻¹ at pH 6.0.

Reactivation of acetyl-chymotrypsin was monitored con-
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from the chymotrypsin first-order reactivation the E1 where be to a corresponding experiment. the appearance of the enzyme, respectively, and k is the first-order reactivation rate constant. The concentration of reactivated chymotrypsin at any given time can be calculated from the relationship:

\[ (E_n) = (E_T)(1 - e^{-kt}) \]  

where \( (E_n) \) represents total enzyme concentration.

When the appearance of \( E_n \) is followed in a pH stat by using excess substrate, S, the rate of formation of product, P, is given by the expression

\[ \frac{d(P)}{dt} = k_{cat}(E_T)(1 - e^{-kt}) \]  

or

\[ P = k_{cat}(E_T)t - [k_{cat}(E_T)/k](1 - e^{-kt}) \]  

in which \( k_{cat} \) is the catalytic rate constant.

Because the formation of \( P \) is stoichiometrically related to the uptake of NaOH in the hydrolysis of AcTyrEt, Eq. 4 may be rewritten as follows:

\[ Mu/(V + u) = k_{cat}(E_T)t - [k_{cat}(E_T)/k](1 - e^{-kt}) \]  

where \( M \) is the molarity of the titrant (NaOH), \( V \) is the initial reaction volume, and \( u \) is the volume of the titrant added in time (t). However, the continuous addition of the titrant and the hydrolysis of AcTyrEt would result in a net decrease in the initial saturating concentration of substrate, \( S_0 \). This dilution and depletion of substrate would lead to a decrease in the rate of AcTyrEt hydrolysis.

After correcting for substrate depletion and dilution the final expression for the uptake of NaOH due to the reactivation of acetyl-chymotrypsin may be represented as follows:

\[ f = \ln([V + u]/V) + (K_m/M)\ln[V(S_0)/V(S_0) - uM]] = [(E_T)/M]k_{cat}t - (k_{cat}/k)(1 - e^{-kt}) \]  

where \( K_m \) is the Michaelis-Menten constant for AcTyrEt, and \( S_0 \) is the initial substrate concentration. A mathematical derivation of this expression is described in the Appendix.

The data in Fig. 2 (u versus t) are replotted as f versus t in Fig. 3. However, though \( f \) compensates for substrate dilution and depletion, the qualitative nature of the reaction curve remains essentially unchanged. An experimentally determined \( K_m \) value of 0.7 mM was used to calculate \( f \) values.

An examination of Eq. 6 indicates that a plot of \( f \) versus \( t \) should yield the relationship shown in Fig. 3 (insert), from which the first-order reactivation rate constant, as well as the catalytic rate constant \( k_{cat} \) may be readily obtained. Treatment of our data in this manner provided a reactivation rate constant with a value of \((0.2 \pm 0.015) \text{ min}^{-1}\), and a catalytic rate constant of \((1200 \pm 60) \text{ min}^{-1}\).

These results obtained by studying the kinetics of reactivation, however, are in conflict with our kinetic data on deacetylation as followed by the decrease in \(^{14}C\text{acetyl-chymotrypsin} \) concentrations and also with the \( k_{cat} \) value obtained for the unmodified enzyme towards AcTyrEt as a substrate by
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FIG. 3. NaOH uptake profile for reactivating monoacetyl-chymotrypsin (23.3 nM) after correction for substrate and enzyme dilution and for substrate depletion. f values are computed from the data in Fig. 2. Insert: the expected relationship of the function f with respect to time according to Eq. 6 and its utilization for the determination of the reactivation rate constant k and the catalytic rate constant k_{cat}.

FIG. 4. Rates of deacetylation and reactivation of mono-[14C]acetyl-chymotrypsin at pH 6.0. The relative esterase activity of the reactivating enzyme towards AcTyrEt (O--O) is derived from the data in Fig. 3. Computer generated values for df/dt from the experimental data in Fig. 3 are plotted as percent of $[k_{cat}(E_T)/M]$. [14C]acetate released from the acetylated protein (●--●) at pH 6.0 is computed from the data in Fig. 1. Data at 240 min (100 percent of [14C] released) not shown.

conventional procedures (11) under identical conditions. Thus, while deacetylation proceeded with a first-order rate constant of 0.02 min⁻¹, reactivation occurred 10-fold faster with a rate constant of 0.2 min⁻¹. Further, the reactivated enzyme was found to have a k_{cat} value of 1200 min⁻¹ compared to a value of 945 min⁻¹ for the unmodified chymotrypsin. However, this enhanced rate of catalysis gradually decreased (Fig. 3) to the true k_{cat} value of approximately 950 min⁻¹.

A first differentiation of the function f with respect to time readily provides a measure of the rate of AcTyrEt hydrolysis, and the value of this derivative relative to [k_{cat}(E_T)/M] may be expressed as esterolytic activity relative to that of the native enzyme, where k_{cat} is the catalytic rate constant of unmodified chymotrypsin with AcTyrEt as substrate determined by conventional procedures. Fig. 4 shows the experimentally observed rates of deacetylation and reactivation of monoacetyl-chymotrypsin. It is evident from these results that the enzyme regains 100% of its activity while it is still 90% acetylated. This observation has previously been reported (6) and confirmed (2). These observations further reveal that during deacetylation the enzyme acquires a transient reactivity towards AcTyrEt which is considerably greater than the catalytic capacity of unmodified chymotrypsin. This enhanced catalytic activity, however, is abolished upon complete deacetylation and the enzyme returns to its normal catalytic activity towards AcTyrEt.

The results presented thus far are hard to reconcile with a simple first-order deacetylation process resulting in concomitant reactivation as depicted in Eq. 1. Consequently, we undertook to analyze our observations according to the following deacetylation mechanisms:

$$E_i \xrightarrow{k_1} E_a \xrightarrow{k_2} E_n + \text{acetate} \quad [7a]$$

$$E_i \xrightarrow{k_1} E_a \xrightarrow{k_3} E_n + \text{acetate} \quad [7b]$$

In both instances, we postulated an acetylated enzyme intermediate E_a which is esterolytically active. Such an intermediate would presumably arise from an acetyl transfer step which would unblock the catalytic center of the enzyme. Eq. 7a demands E_a to be a mandatory intermediate, while Eq. 7b implicates it in an alternate deacetylation mechanism. Conventional kinetic considerations, similar to those utilized for the derivation of Eqs. 4 and 6 provided the following expressions representing base uptake by reactivating acetyl-chymotrypsin according to mechanisms [7a] and [7b], respectively.

$$f = [(E_T)/M][k_{cat}^1 + [(k_1k_{cat} - k_2k_{cat})/k_1(k_2 - k_1)] \times (1 - e^{-k_1t})] + [(k_1(k_{cat} - k_{cat}))/k_2(k_2 - k_1)] \times (1 - e^{-k_2t})] \quad [8a]$$

$$f = [(E_T)/M][k_{cat}^1 + [(k_1k_{cat} - k_2k_{cat})/k_1(k_2 - k_1)] + k_2k_{cat}]/(k_1 + k_2)[1 - e^{-(k_1 + k_3)t}) + [(k_1(k_{cat} - k_{cat}))/k_2(k_2 - k_3)] \times (1 - e^{-k_3t})] \quad [8b]$$

An exact kinetic determination of the release of [14C]acetate would necessarily provide the best means of distinguishing between mechanisms [7a] and [7b], because the catalytic parameters (k_{cat} and k_{m}) of the transient intermediate (E_a) cannot be determined with sufficient accuracy to exactly predict f as a function of time. Furthermore, the experimental procedure we utilized in determining the release of acetate from acetyl-chymotrypsin did not provide us with data precise enough to distinguish between these two mechanisms. Consequently, we decided to interpret our results in accordance with the simpler deacetylation mechanism [7a], which may be described as a special case of [7b] when k_1 > k_2.

The expected and observed rates of [14C]acetate release, as well as of the levels of relative esterase activity, are illustrated in Fig. 5. We utilized the graphical procedure shown in Fig. 3 (insert) to estimate the reactivation rate constant k_1, with the added approximation that k_1 >> k_2. The value of the deacetylation rate constant (k_2) was derived from the release of [14C]acetate (Fig. 1) and from a first-order plot of the decrease in relative esterase activity to 100% from Fig. 4 (not shown). By using the determined k_{cat} value of chymotrypsin towards AcTyrEt (945 min⁻¹), we computed the value of k_{cat} for the transient intermediate E_a to be of the order of 1500 min⁻¹.

Based upon these parameters, curve A and B (Fig. 5) are the
expected computer drawn profiles for the levels of esterase activity and $[^{14}C]$acetate release, respectively. Our results, on interpretation in accordance with the two-step deacylation mechanism [7a], may be summarized as follows:

$$E_t\left(0.2+0.015\text{min}^{-1}\right) \xrightarrow{k_1} E_a\left(0.02+0.0075\text{min}^{-1}\right) \xrightarrow{k_2} E_n + \text{acetate}$$

$$k'_{\text{cat}} = 1500 \pm 100 \text{ min}^{-1}$$

$$k'_{\text{cat}} = 945 \pm 15 \text{ min}^{-1}$$

It is, however, apparent to us that because $E_n$ can acylate and deacetylate with respect to the N-acetyl-tyrosyl moiety (it is esterolytically active towards AcTyrEt) without a mandatory acyl transfer (the transfer site presumably being occupied by $[^{14}C]$acetate) mechanism [7b] best represents the possible sequence of events in the deacylation of monoacetyl-chymotrypsin.

We wish to emphasize that these mechanisms appear to be applicable to only the case of monoacetyl-chymotrypsin, because reactivation of other acyl enzymes such as cinnamoyl- and indole-acyroyl-chymotrypsin occur concomitantly with the deacylation process. However, in view of the recent evidence in the literature favoring the possibilities for a two-step deacylation of furoylacyroyl-chymotrypsin derivatives (12) and the duality in the nature of trifluoromethylbenzenesulphonyl-chymotrypsin (13), we feel that our observations might well have general applicability to the mechanism of chymotryptic catalysis.

APPENDIX

The rate of formation of acetyl-tyrosine, $P$, as a consequence of AcTyrEt hydrolysis by reactivating chymotrypsin is depicted in Eq. 3. This expression assumes the reaction to proceed at constant volume, $V$, in the presence of excess substrate concentration ($S_0$). Under these conditions, according to Eq. 3,

$$\frac{d(P)}{dt} = K_{\text{cat}}(E_t)(1-e^{-k't})$$

The formation of $P$ is stoichiometrically related to the addition of titrant (NaOH) to the reaction mixture so that

$$P = Mu/(V + u)$$

at all times in which $M$ = molarity of titrant, $V$ = reaction volume at zero time, and $u$ = volume of titrant added in any time $t$. Under experimental conditions, there is a cumulative addition of the titrant to the reaction mixture, so that $(E_T)$ is no longer constant. Thus, if $(E)$ represents the total enzyme concentration at any time $t$,

$$(E) = (E_T)\sqrt{V/(V + u)}.$$ [iii]

Then from [ii]

$$(E) = (E_T)(1 - (P)/M)].$$ [iv]

The addition of titrant also causes a dilution of the initial saturating concentration of the substrate, $(S_0)$, which is further decreased due to its continued hydrolysis by reactivated chymotrypsin. Thus, the total concentration of the substrate and its product at any time $t$ may be represented as

$$(S')_t = (S_0)\sqrt{V/(V + u)}$$ [v]

when $(S_0)$ is the initial total substrate concentration. The concentration of unutilized substrate at any time may then be represented by

$$(S) = [(S_0)\sqrt{V/(V + u)} - (P)]$$ [vi]

which may be expressed as

$$(S) = [(S_0)[1 - (P)/M]] - (P)$$ [vii]

The rate equation [i] on correction for substrate and enzyme dilution, and for substrate hydrolysis, may be expressed as

$$\frac{d(P)}{dt} = [K_{\text{cat}}(S)/(S) + K_m][E_T][1 - (P)/M][1 - e^{-k't}]$$ [viii]

where $K_m$ is the Michaelis-Menten constant for the chymotryptic hydrolysis of AcTyrEt. Or

$$[1 + K_m(S)]/[1 - (P)/M]d(P) = [K_{\text{cat}}(E_T)(1 - e^{-k't})]dt.$$ [ix]

Substituting for $(S)$ from [vii], integrating with respect to $(P)$
and $t$, and further substituting for $(P)$ from [ii] we obtain Eq. 6

$$f = \ln[(V + u)/V] + \frac{(K_m/M)}{[ii]} \ln[V(S_0) - uM]$$

$$= \frac{[(E_T)/M][k_{cat}t - (k_{cat}/k)(1 - e^{-kt})]}{[x]}$$

which relates the volume of base uptake ($u$) as a function of time as a consequence of chymotrypsin reactivation.

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