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The Kinetic Equivalence of Reactivated Phenylmethanesulfonyl-α-Chymotrypsin and Native α-Chymotrypsin. A Reexamination*

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ABSTRACT: The enzyme resulting from reactivation of phenylmethanesulfonyl-α-chymotrypsin was reported to differ from native α-chymotrypsin in its kinetic properties [Gold, A. M., and Fahrney, D. (1964), *Biochemistry 3*, 783]. This conclusion has been found to be in error.

The reactivated enzyme and the native enzyme have the same K_M (2.8×10^{-4} M) and k_{cat} (1.5 min^{-1}) with the substrate *N*-glutaryl-L-phenylalanine *p*-nitroanilide. The rate-limiting step in the hydrolysis of this substrate is the formation of an acyl enzyme. Measurements of the rate of hydrolysis of *p*-nitrophenyl acetate indicate that the enzymes also have identical rate constants for hydrolysis of the corresponding acetyl enzymes (0.28 min^{-1}). It must be concluded that no differences exist between the kinetic properties of the native and reactivated enzymes and that the low specific activity observed for the reactivated enzyme is the result of partial denaturation during reactivation.

Phenylmethanesulfonyl-α-chymotrypsin can be reactivated by subjecting the protein to treatment with acid followed by incubation in neutral solution (Gold and Fahrney, 1964). This process appears to consist of a series of chemical reactions. Under the influence of acid the *O*-phenylmethanesulfonylserine residue in the active site (Gold, 1965) first undergoes cyclization to an oxazoline which then rapidly hydrolyzes to the corresponding *O*-acylserine derivative. This intermediate is isomeric with α-chymotrypsin but differs in having an ester linkage, rather than the normal peptide bond, between the aspartic acid and serine residues of the active site. In neutral solution the intermediate undergoes an *O,N*-acyl shift, yielding a protein having the same primary structure as α-chymotrypsin but only 60–70% of its enzymic activity in an assay using *N*-acetyl-L-tyrosine ethyl ester. The reactivated enzyme was found to have the same apparent equivalent weight as native α-chymotrypsin from measurements of the extent of reaction with [7-^{14}C]-phenylmethanesulfonyl fluoride. On the basis of this evidence it was concluded that the reactivated enzyme is not identical with α-chymotrypsin in its kinetic properties and therefore also differs in some details of structure. Experiments reported in this paper negate this conclusion and indicate that the reactivated enzyme is probably identical with native α-chymotrypsin, at least in its kinetic properties.

Experimental Section

Protein concentrations were calculated from the absorbance at 282 mμ using the extinction coefficient 2.07 ml mg^{-1} for a 1-cm light path.

Reactivation of phenylmethanesulfonyl-α-chymotrypsin was carried out in 0.040 M NaCl solution at pH 2.0 (HCl) as described previously (Gold and Fahrney, 1964). The protein concentration was 0.15 mg ml^{-1}. When it was necessary to isolate the product the reaction was carried out on a 1-l. scale. After reactivation was complete, the solution was acidified to pH 3.0 with HCl and percolated through a column containing 10 g of sulfoethyl cellulose (Bio-Rad) in its sodium form. The substantial absence of protein in the effluent was confirmed by periodically measuring the absorbance at 282 mμ. When all the protein had been

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adsorbed to the column it was eluted in a small volume by passing through 0.2 M sodium citrate buffer (pH 5.3). The citrate was removed by exhaustive dialysis against 1 mM HCl and the solution was lyophilized to dryness. All operations except lyophilization were conducted in the cold.

Enzyme assay with *N*-glutaryl-L-phenylalanine *p*-nitroanilide was carried out at 25° in 0.050 M Tris-HCl (pH 7.6), 0.020 M $CaCl_2$, as described previously (Gold, 1965). The substrate concentration was 1.33 mM. The K_M for this substrate was determined under the same conditions; however, the change in optical density at 410 mμ was followed over a period of time by incubating the solution in a cuvet in the constant temperature bath and transferring it to a colorimeter for periodic absorbance measurements. The K_M and V_{max} were determined graphically from a Lineweaver–Burk plot.

Reaction with [7-^{14}C]Phenylmethanesulfonyl Fluoride. About 2.5 mg of enzyme dissolved in 1.75 ml of 0.03 M sodium phosphate buffer (pH 7.0) was treated with 0.10 ml of 2.0 mM ^{14}C-labeled inhibitor in 2-propanol (sp act. 1.20 mc/mmole). After standing at room temperature for 30 min the solution was dialyzed exhaustively against 1 mM HCl in the cold.

Radioactivity measurements were carried out with a liquid scintillation counter. The phosphor solution was prepared with 0.50 ml of aqueous protein solution, 0.50 ml of hyamine hydroxide (1 M in methanol), 5.00 ml of absolute ethanol, and 10.0 ml of a solution of 0.50% 2,5-diphenyloxazole and 0.030%1, 4-bis-2-(5-phenyloxazolyl)benzene in toluene. Counting rates were determined with a statistical error of <1%. Efficiency was determined with commercial [^{14}C]-benzoic acid of known activity.

Reaction with p-nitrophenyl acetate was carried out at 25° in 0.050 M sodium phosphate buffer (pH 7.4) containing 5% acetonitrile, 1.0×10^{-4} M *p*-nitrophenyl acetate, and about 1 mg/ml of protein. The protein, in 1.00 ml of 1 mM HCl, was rapidly added to the other components in 2.00 ml of solution in a spectrophotometer cuvet and the absorbance at 400 mμ was automatically recorded for a period of several minutes using a Zeiss PMQII spectrophotometer equipped with a logarithmic recorder. No more than 5 sec usually elapsed between the time of addition of enzyme and the start of recording. The linear portion of the resulting curve was extrapolated to zero time to determine the "burst." Blanks were carried out without enzyme to determine the absorbance of the solution at the initial time and the rate of spontaneous hydrolysis of the substrate.

Results

Phenylmethanesulfonyl-α-chymotrypsin was reactivated by the same procedure described previously (Gold and Fahrney, 1964) and assayed by another, more convenient, method using *N*-glutaryl-L-phenylalanine *p*-nitroanilide as substrate (Erlanger *et al.*, 1964, 1966). The reactivated enzyme had a specific activity which varied from 50 to 75% of that of the native enzyme, in substantial agreement with the results obtained using *N*-acetyl-L-tyrosine ethyl ester as substrate. However, in nine separate experiments the K_M for the reactivated enzyme was the same as that of native α-chymotrypsin, $2.8 \pm 0.2 \times 10^{-4}$ M.

In order to determine whether the reactivated enzyme differed from native enzyme in its specific rate constant or merely contained some denatured protein it was necessary to measure the concentration of active sites as well as the enzymic activity of particular preparations. This was accomplished by a modification of the method used previously, determining the extent of reaction of the protein with [7-^{14}C]phenylmethanesulfonyl fluoride. The reactivated enzyme was first concentrated by adsorption on a column of sulfoethyl cellulose followed by elution with a buffer of high ionic strength. After removal of the buffer salt by dialysis the protein was isolated by lyophilization. Reaction with the ^{14}C-labeled inhibitor was carried out at a protein concentration ten times greater than used previously and the radioactivity of the inhibited enzyme, which retained <1% of its original enzymic activity, was determined by means of liquid scintillation counting. The results are shown in Table I. Reactivated chymotrypsin is indistinguishable from the native enzyme by these measurements.

TABLE I: Specific Rate Constants for Hydrolysis of *N*-Glutaryl-L-phenylalanine *p*-Nitroanilide.

Enzyme	V_{max}[a] (μmoles min^{-1} mg^{-1})	Sp Act. (μc/mg)	k_{cat}[b] (min^{-1})
Native α-chymotrypsin	0.441	0.0400	1.50
Reactivated α-chymotrypsin	0.399	0.0367	1.48

[a] Enzyme activity was determined in the standard assay. V_{max} was calculated using $K_M = 2.8 \times 10^{-4}$ M. [b] k_{cat} was calculated from the equation $k_{cat} = V_{max}/E_0$. Specific activity 1.20 μc/μmole was used for the [7-^{14}C]-phenylmethanesulfonyl fluoride.

More evidence to support this conclusion was obtained with another substrate, *p*-nitrophenyl acetate. An advantage of using this substrate is that the hydrolysis proceeds in two kinetically distinguishable stages: rapid acetylation of the enzyme with the release of *p*-nitrophenol and subsequent hydrolysis of the acetyl enzyme (Hartley and Kilby, 1954). When the reaction is followed by measuring the absorbance of *p*-nitro-

phenol at 400 mμ there is an initial "burst" of *p*-nitrophenol, which is related to the amount of enzyme present, followed by a slower increase which is a measure of the rate at which the acetyl enzyme hydrolyzes (Kézdy and Bender, 1962; Kézdy *et al.*, 1965). Thus, it is possible to obtain an estimate of the concentration of active sites and their catalytic efficiency in the same experiment. Repetitive experiments under a given set of conditions should give a constant ratio of steady-state rate to "burst," regardless of the amount of enzyme used each time.

This procedure was applied to the enzyme under consideration. Six measurements with native α-chymotrypsin gave an average value for the ratio of 0.282 ± 0.006 min^{-1}, while an equal number of determinations with reactivated enzyme gave an average value of 0.283 ± 0.006 min^{-1}. Under the conditions of this experiment, this ratio should be equal to the rate constant for hydrolysis of the acetyl enzyme; the corresponding constant calculated for pH 7.4 from the data of Kézdy and Bender (1962) is 0.23 min^{-1}.

Discussion

These experiments clearly indicate that there is no kinetic difference between the native and reactivated enzymes, contrary to our original conclusion. The substrate *N*-glutaryl-L-phenylalanine *p*-nitroanilide reacts with α-chymotrypsin without producing an initial "burst" of *p*-nitroaniline; consequently, the first step in the hydrolysis, acylation of the enzyme with an *N*-glutaryl-L-phenylalanyl group, must be the rate-limiting step. The rate-limiting step for the hydrolysis of *p*-nitrophenyl acetate is known to be the reaction of the acetyl enzyme with water. By using these two substrates the catalytic efficiency of the reactivated enzyme can be compared to that of the native enzyme in each of the reactions which make up the over-all hydrolysis process. The identity of the K_M values determined for *N*-glutaryl-L-phenylalanine *p*-nitroanilide with the two enzymes indicates that no differences exist in the substrate-binding sites. The low specific activity of reactivated α-chymotrypsin must be due to side reactions or denaturation of a portion of the enzyme during reactivation.

It is probable that the earlier work gave misleading results because of losses of denatured protein during dialysis of the very dilute (0.15 mg/ml) [7-^{14}C]phenylmethanesulfonyl protein against solutions of low ionic strength. Similar experiments with native chymotrypsin tend to show erratic losses of protein. This difficulty is overcome in the present work by the use of much higher protein concentrations in the reaction with the radioactive sulfonyl fluoride.

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