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Thermal and pH stability of "β-benzyme"

(artificial enzyme/chymotrypsin)

V. T. D'SOUZA, X. L. LU, R. D. GINGER, AND MYRON L. BENDER*

Chemistry and Biochemistry Departments, Northwestern University, Evanston, IL 60201

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ABSTRACT **The thermal and pH stability of "β-benzyme," an artificial chymotrypsin based on β-cyclodextrin, has been studied and compared with the stability of real chymotrypsin. Artificial chymotrypsin is vastly superior to real chymotrypsin with regard to both temperature and pH stability. The reasons for this increased stability are discussed.**

Although enzymes are difficult to surpass for efficiency, versatility, and economy there are limitations that make them inadequate for practical and industrial uses. One of the most important problems is their instability at high temperature and pressure, under mechanical stress, in organic solvents, and under detergent conditions. Enzymes in general can undergo two kinds of inactivation, reversible and irreversible.

Enzymes are critically dependent on their conformation for their catalytic activity. Their conformational stability is determined by hydrogen bonding, hydrophobic interactions, van der Waals interactions, configurational entropy, hindered rotation, permanent dipole interactions, electrostatic effects (including those of abnormal acidic and basic groups), and the interaction of proteins with water (1). The forces that disturb these interactions tend to change the conformation, to unfold the protein, and thus inactivate the enzyme reversibly. The removal of such forces under certain conditions can potentially refold the protein and thus reproduce the active enzyme (1). This has been demonstrated by the reduction of the disulfide link and reoxidation of the resulting sulfhydryl groups in the enzyme ribonuclease (2). Some of the agents that bring about such changes are temperature, organic solvents, urea, and ionic strength.

Irreversible inactivation of chymotrypsin is brought about by disruption of the protein by cleavage of peptide linkages by the proton, by the hydroxide ion, or by the enzyme itself ("cannibalistic reaction") (3). It is thus important to study the stability of the enzyme and be able to overcome this deficiency. One of the important methods for increasing the stability of the enzyme is to immobilize the enzyme by covalently attaching it to a surface and thus prevent its intermolecular interactions and thereby minimize the cannibalistic reaction (4).

Another method to avoid inactivation is to synthesize an artificial enzyme that is not susceptible to inactivation by the above-mentioned processes. "β-Benzyme," an artificial chymotrypsin based on β-cyclodextrin, has been prepared. Its kinetics (k_{cat}/K_m) is equivalent to that of real chymotrypsin (5), and it has a turnover similar to that of a real enzyme (6).

With respect to its stability, it is important to note that artificial chymotrypsin contains no amino acids, whereas real chymotrypsin contains 245 amino acids. The function of 242 of the amino acids in the real enzyme is to achieve a precise and correct conformation through hydrogen bonding, while the other 3 amino acids are involved in catalysis. When one of the hydrogen bonds between these noncatalytic amino acids is disrupted, the conformation is changed and the enzyme activity diminishes. The disruption of the hydrogen bonds can be caused by extremes of temperature and pH, usually high temperature or high alkalinity. Any perturbation of the conformation that depends on amino acid interactions should be much less for the artificial enzyme than for the real enzyme. This has been observed in both thermal stability and pH stability (Figs. 1 and 2).

EXPERIMENTAL

Materials. The synthesis of the artificial chymotrypsin (β-benzyme) has been described elsewhere (5). The product was purified by reverse-phase preparative HPLC, and its purity was confirmed by HPLC, NMR, and thin-layer chromatography. α-Chymotrypsin from Worthington was used without further purification. *p*-Nitrophenyl acetate was obtained from Aldrich and was recrystallized before use. *m*-(*t*-Butyl)phenyl acetate was synthesized from the corresponding phenol and acid chloride and recrystallized before use. All buffer solutions were prepared from doubly distilled water and analytical reagent grade chemicals. The pH values of all reaction mixtures and buffers were determined by pH meter (Altex model 3500 digital pH meter; Beckman).

Kinetic Measurements. The kinetics of all the reactions were observed spectrophotometrically with a Cary 219 recording spectrophotometer (Varian) equipped with a thermostated cell compartment and interfaced with an Apple II Plus computer. The kinetics of α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate was followed at 400 nm in pH 8 sodium phosphate buffer. In a typical experiment a 50-μl sample of a stock solution of α-chymotrypsin (50 mg/ml) in pH 4 sodium acetate buffer and a 50-μl sample of *p*-nitrophenyl acetate solution (20 mM in acetonitrile) were added at the same time to 2.9 ml of buffer solution previously equilibrated to the desired temperature in the thermostated cell compartment and recording was commenced as soon as possible.

The kinetics of β-benzyme-catalyzed hydrolysis of *m*-(*t*-butyl)phenyl acetate was followed at 288 nm in pH 10.7 sodium carbonate buffer. In a typical experiment a 5-μl sample of *m*-(*t*-butyl)phenyl acetate (20 mM in acetonitrile) was added to 3 ml of carbonate buffer containing a 50-μl sample of benzyme stock solution (2 mM) and equilibrated to the desired temperature in the thermostated cell compartment, and recording was commenced immediately. In the series of experiments to study the stability of the enzyme at various pH values, 100-μl samples of the different pH stock solutions (0.7 mM) were added to the reaction mixture at pH 10.7.

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*To whom reprint requests should be addressed.

RESULTS AND DISCUSSION

Both the temperature dependence of k_{cat} (Fig. 1) and the pH dependence of $k_{denaturation}$ (Fig. 2) for the real enzyme differ from those for the artificial enzyme. The artificial enzyme is more stable than the real enzyme, because the artificial enzyme contains no amino acids, whereas the real enzyme contains 245 amino acids.

Since the conformation is primarily determined by amino acids in the real enzyme, the temperature dependence of the stability of the real enzyme is markedly different and less favorable than that of the artificial enzyme. Fig. 1 shows that real chymotrypsin has a temperature maximum around 45°C and above 55°C the protein begins to precipitate and is rendered inactive, whereas the activity of the artificial enzyme keeps increasing to at least 80°C. This reversible inactivation of chymotrypsin can be explained on the basis of conformation of the enzyme. The three groups involved in the catalysis—namely, the hydroxyl group of serine-195, the imidazolyl group of histidine-57, and the carboxylate ion of aspartate-102—are positioned in a precise manner to form the catalytic triad. The interatomic distances are 2.8 Å between Ser-195 O^{γ} and His-57 $N^{\epsilon 2}$; and 2.65 Å between His-57 $N^{\delta 1}$ and Asp-102 $O^{\delta 1}$ (7). This positioning is the result of the tertiary structure of the enzyme. Elevation in temperature perturbs the forces that determine this precise conformation and thus inactivates the enzyme. However, the catalytic triad of the artificial enzyme—namely, the hydroxyl group of cyclodextrin and the imidazolyl group and the carboxylate ion of the *o*-imidazolylbenzoic acid moiety—are brought together by covalent attachment, which is resistant to perturbation by elevated temperatures. Cyclodextrin complexes are known to be stable up to the degradation temperature (200°C) of cyclodextrin (8). Thus, it is reasonable to expect the activity of the artificial enzyme to increase up to 200°C.

Irreversible inactivation of chymotrypsin as a function of pH between 6 and 13 has long been known to be complicated (9), showing a "roller coaster" profile, involving a bell-shaped curve around neutrality with a maximum around pH 8 and a minimum around pH 10.5. A further increase in pH leads to greater inactivation, as can be seen in Fig. 2. It has been shown that the denaturation around neutrality shows

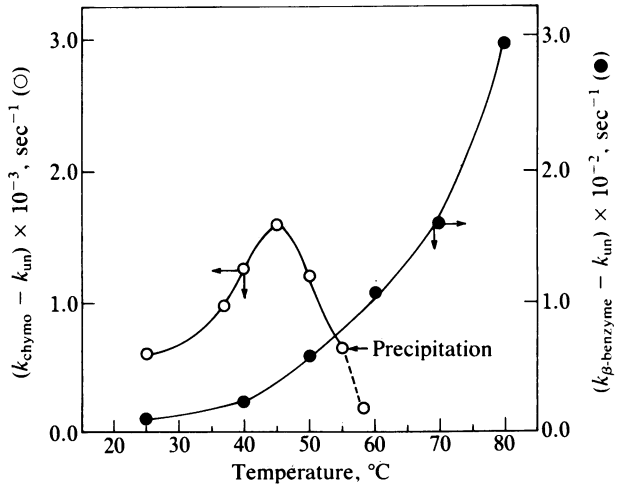

FIG. 1. Thermal stability of real (○) and artificial (●) chymotrypsins. k_{un} refers to the rate for the uncatalyzed reaction.

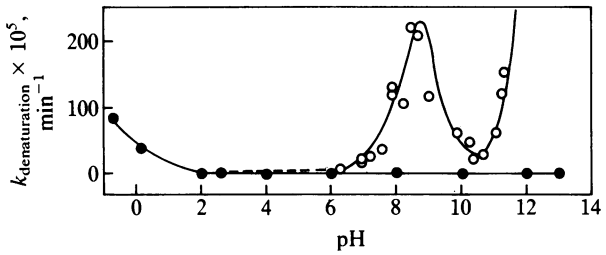

FIG. 2. pH stability of real (○) and artificial (●) chymotrypsins.

second-order kinetics with respect to enzyme. This has been attributed to the cannibalistic reaction of the enzyme since peptide bonds of two of the amino acids (tyrosine and tryptophan) of chymotrypsin are natural substrates for chymotrypsin. However, since the artificial enzyme is made up of glucose units, which are not substrates for the artificial enzyme, such inactivation is absent in the artificial enzyme (Fig. 2). At high pH the inactivation of chymotrypsin has been shown to be first-order in enzyme and first-order in hydroxide ion, indicating the reaction is one of hydroxide ion with the enzyme (3). Thus, at high pH the hydroxide ion attacks the peptide linkage of the protein to inactivate the enzyme. The artificial enzyme, on the other hand, contains no peptide linkages and instead contains α1-4 glycosidic linkages, which are resistant to hydroxide ion attack (10). Thus, as can be seen from Fig. 2, the artificial enzyme is very stable at high pH. The α1-4 glycosidic linkages are susceptible to attack by protons, and hence the artificial enzyme is inactivated at low pH (Fig. 2). However, the conditions (high acidity and high temperature) required for such cleavage are so severe that the practical use of the artificial enzyme would not be hindered. In fact, cyclodextrins are known to have a half-life of 48 hr at pH −0.133 and 40°C (10).

Thus, with regard to both temperature and pH, the stability of artificial chymotrypsin is vastly superior to that of real chymotrypsin. One of the greatest limitations of natural chymotrypsin, its instability, can thus be overcome by artificial chymotrypsin.

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1. Lumry, R. & Biltonen, R. (1969) in *Structure and Stability of Biological Macromolecules*, eds. Timasheff, S. N. & Fasman, G. D. (Dekker, New York), pp. 65–205.
2. Schachman, H. K. & Edelstein, S. J. (1966) *Biochemistry* **5**, 2681–2705.
3. Wu, H.-L., Wastell, A. & Bender, M. L. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4116–4117.
4. Wu, H.-L., Lace, D. A. & Bender, M. L. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4118–4119.
5. D'Souza, V. T., Hanabusa, K., O'Leary, T., Gadwood, R. C. & Bender, M. L. (1985) *Biochem. Biophys. Res. Commun.* **129**, 725–731.
6. Bender, M. L., D'Souza, V. T. & Lu, X. (1986) *Trends Biotechnol.* **4**, 132–135.
7. Tsukada, H. & Blow, D. M. (1985) *J. Mol. Biol.* **184**, 703–712.
8. Szejtli, J. (1982) *Cyclodextrins and Their Inclusion Complexes* (Akademiai Kiado, Budapest), pp. 101–137.
9. Bender, M. L., Clement, G. E., Kézdy, F. J. & d'Heck, H. (1964) *J. Am. Chem. Soc.* **86**, 3680–3690.
10. Bender, M. L. & Komiyama, M. (1978) *Cyclodextrin Chemistry* (Springer, New York), p. 9.