THE STABILITY OF CHYMOTRYPSIN CROSS-LINKED WITH FORMALDEHYDE

Leo J. Saidel, Stanley Leitzes and William H. Elfring, Jr.

Department of Biochemistry
The Chicago Medical School
Chicago 12, Illinois

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The stability of crystalline α-chymotrypsin (CT) activity to the denaturing effects of heat and 8 M urea is increased by the reaction of the enzyme with HCHO. The conditions used for the HCHO reaction introduce covalent cross-links into protein molecules between amino groups on the one hand and phenol, imidazole, indole, sulfhydryl, amide, guanido or other amino groups on the other hand (Fraenkel-Conrat and Olcott, 1948). A reasonable explanation for the stability of the CT after reaction with HCHO would be the formation of covalent cross-links which lock the molecule into a conformation capable of enzyme activity yet stabilized against denaturing forces.

Experimental

Materials -- Most of the experiments were done with Worthington, three times crystallized, salt free α-chymotrypsin Lot CDI 6077. Similar results were obtained with another preparation of crystallized α-chymotrypsin obtained from Kuster Laboratories. Urea was recrystallized from water-methanol.

Methods -- CT activity was measured at pH 7 and 25° with tyrosine ethyl ester as substrate by the method of Schwert and Takenaka (1955). The initial rate of hydrolysis was measured at 237 mμ with a time driven recording spectrophotometer. CT concentration in the assay mixture was 8 μg/ml.

Stability to heat was determined by measuring the CT activity surviving 10 minutes exposure to 55° in 0.05 M sodium phosphate buffer pH 7 at 10 µg/ml CT concentration.

Stability to urea was determined by measuring CT activity in 8 M urea held at pH 5.5 by 0.05 M sodium phosphate buffer.

The ninhydrin color reaction essentially as developed by Moore and Stein (1954) was used to estimate reactive amino groups.

HCHO Reaction -- HCHO and CT were reacted at 25° up to 7 days in 0.05 M sodium phosphate buffer, pH 7, containing 2 mg/ml of CT and 20 mg/ml (0.67 M) of HCHO. In some experiments 0.09 M sodium β-phenylpropionate or sodium acetate was also present. At time intervals diluted reaction mixtures were tested for residual activity and for stability by the methods indicated above. The final concentration of the HCHO in the assay mixture was 0.08 mg/ml. HCHO concentration up to 0.4 mg/ml could be added at the start of assay without appreciably affecting the activity. The final concentration of β-phenylpropionate or sodium acetate in the assay mixture was 0.36 mM. At this concentration the inhibition by β-phenylpropionate was about 12%. Sodium acetate had no inhibitory effect.

Result and Discussion

The effects of HCHO upon CT activity and heat stability in the absence and presence of β-phenylpropionate are presented in Fig. 1. Unlike the specific inhibitor, β-phenylpropionate, sodium acetate at the same ionic strength provided no protection of CT activity against the action of HCHO, nor did it alter the increase in stability.

The stability to urea was determined only with CT treated with HCHO for five days. If the activity of freshly dissolved CT assayed at pH 5.5 in the absence of urea was assigned a reference value of 100%, the HCHO treated CT, assayed under the same conditions, gave a value of 25%. However, if the assay was done at the same pH in 8 M

urea, freshly dissolved CT gave a value on the same reference scale of 4.7% and HCHO treated CT a value of 29%.

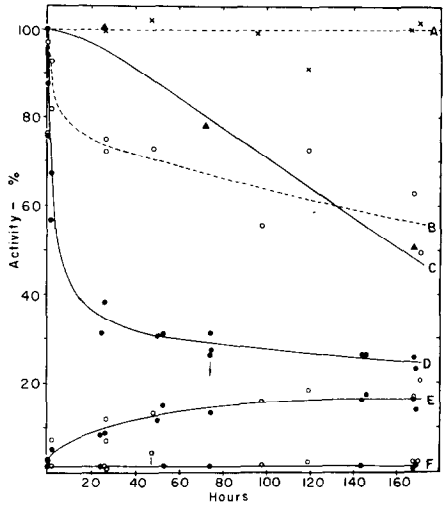

Fig. 1. The effect of HCHO on the activity and stability of CT. All residual activities are compared with that of CT dissolved in corresponding solvent and assayed immediately. All points after zero time represent single determinations.

Controls containing neither β-phenylpropionate nor HCHO: unheated, curve C (▲); heated, curve F (●). Controls containing β-phenylpropionate but no HCHO: unheated, curve A (X); heated, curve F (0). Reaction with HCHO in the absence of β-phenylpropionate: unheated, curve D (●); heated, curve E (●). Reaction with HCHO in the presence of β-phenylpropionate: unheated, curve B (0); heated, curve E (0).

These results demonstrate that the resistance of CT to inactivation by heat and urea, to further inactivation by HCHO and possibly to autolysis all increase as the reaction with HCHO proceeds. The β-phenylpropionate retards inactivation by HCHO and autolysis without appreciably affecting stability to heat treatment. Perhaps the simplest explanation is that of the many reactions of HCHO with CT, those which occur at the active site inactivate the enzyme whereas certain others stabilize the enzyme. Combination of the inhibitor, β-phenylpropionate, with the active site protects it against inactivation without affecting stabilization. The difficulty with this explanation is that of those molecules which undergo stabilizing reactions, a

greater fraction should be protected by β-phenylpropionate against inactivation reactions, i.e., the heat stability should increase more in the presence of β-phenylpropionate than in its absence. Such an effect was not evident.

An explanation which appears to fit the data better is that the CT is heterogeneous to begin with or is rapidly made so by the multiple direct reactions with HCHO. The still active CT molecules may be divided into two classes: those which slowly rearrange to an inactive form and those which slowly rearrange to a stabilized form (presumably, but not necessarily, by a cross-linking reaction). The rearrangement to the inactive form is inhibited by β-phenylpropionate; the rearrangement to the stabilized form is not. This hypothesis will be tested by active site assay, and also by attempts to separate inactive CT from stabilized CT.

Although the resistence to heat inactivation produced by the HCHO is taken to be evidence for cross-linking here, the possibility was considered that part of this resistance is attributable to the retardation of heat accelerated autolysis of susceptible bonds blocked by HCHO.

This interpretation appears to be supported by the following observations. When CT was heated 10 minutes at pH 3.6 and 100° (20 μg/ml CT in 0.001 M HCl containing 0.01 M Na_2SO_4) to minimize autolysis as well as reactivation (Aldrich and Balls, 1958), 26% of initial activity remained on subsequent assay at pH 7; when CT which had been treated with HCHO for 6 days and which retained 23% of initial activity was heated under the same conditions, only 1% of initial activity remained. Moreover, the color produced by ninhydrin increased by about 8 leucine equivalents per mole CT when the enzyme was heated at pH 7 but remained about the same when the enzyme was heated at pH 3.6. On the other hand the HCHO treated CT also gave a color increase of about 6 leucine equivalents when heated at pH 7 and

about 5 when heated at pH 3.6. Thus inactivation of HCHO treated CT by heating it at pH 3.6 may have been caused by acid reversal of HCHO cross-linking, which brought about the collapse of the altered molecule as well as the liberation of amino groups.

The stability to urea inactivation remains as an observation which cannot be readily interpreted as increased resistence to autolysis because the urea inactivation is reversible under the conditions chosen (Martin, 1963).

The balance of evidence therefore indicates that cross-linking is an important factor in producing stability. The identification within the fractionated cross-linked products of the particular amino acid residues joined by HCHO (or other cross-linking agents) under various conditions of treatment will be used in the attempt to map the active conformation of some enzymes.

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