ACTIVITY DISTRIBUTIONS IN MODIFIED α-CHYMOTRYPSIN

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α-Chymotrypsin (CT) has been separated, after heat- or radiation treatment, with respect to enzymatic activity by means of sorption chromatography with a chymotrypsin-specific adsorbent. After heat-treatment, in which all molecules had been unfolded, the refolded monomers were found to be indistinguishible from native CT. In irradiated CT a considerable amount of monomeric products that differ from native CT with respect to activity and conformation were found. These results indicate that the primary structure of native CT defines a unique monomeric conformation. Only chemical modifications can therefore give CT-monomers that differ from native CT.

Modification of enzymes can give products, whose activity differs from that of the native enzyme[1]. By sorption chromatography with enzyme-specific adsorbents, the difference in activity can be used, either to prepare pure proteins[2,3] or, as shown here, to study the activity distribution in modified enzyme samples. A suitable specific adsorbent is a competitive inhibitor bound to an insoluble polymer matrix. The inhibitor must still be able to bind enzyme when bound to the matrix. Separations are performed under conditions where the mobility of the enzyme in a column is governed by adsorption only. Then the mobility decreases and the elution volume increases with the distribution coefficient,

$$K_{D_i} = \frac{n K_i}{\sum_i K_i c_i + 1} \qquad (1)$$

where, n, is the number of adsorbent sites per unit column volume; c_i concentration of enzyme which has the association constant, K_i, for the interaction with the adsorbent sites. The different enzyme molecules compete for the same sites, and $K_{D_i} > K_{D_j}$ when $K_i > K_j$. When the enzyme activity increases with K_i, enzyme molecules with high activity will be eluted after molecules with low activity. Under these conditions specific adsorbents can be used to study activity distributions in samples of modified enzyme. Here this method is used to study the heat- and radiation-induced modification of α-chymotrypsin (CT). Mee[4] has shown that the apparent Michaelis-Menten constant increases with radiation dose in unfractionated samples of CT. This indicates the radiation induced formation of modified CT with activity > 0, but different from the activity of native CT.

Soybean trypsin inhibitor (STI) has been found to be a suitable competitive inhibitor for CT[5]. STI was covalently linked to 4 % Sepharose, using a method developed by Axén, Porath and Ernback[6]. It did retain the ability to bind native CT, although the association constant, determined by adsorption measurements[5], is lower than the corresponding constant for interaction of free STI and CT determined by a gel equilibrium dialysis method[7,5] (Table I). This decrease is probably due to steric hindrance of the association reaction by the polymer network.

Stock solutions of CT (Worthington, three times crystallized, salt free and purified by ultrafiltration) were diluted with water and adjusted to the desired pH by addition of 1 M HCl or 1 M NaOH. Solutions were bubbled with nitrogen prior to and during irradiation. This procedure reduces the oxygen content to $< 10^{-6}$ M [5]. Crystalline CT was dried on a vacuum line (4 hr at 0.001 mm Hg) and irradiated under nitrogen atmosphere. The CT-solutions and crystalline CT were

Table I.

The association constants for the interaction of CT with STI and STI-Sepharose, I = 0.25, of which 0.05 due to buffering components and 0.2 to NaCl, 25.0°C. Buffer systems, HAC-NaOH at pH = 5.0, KH_2PO_4-Na_2HPO_4 at pH = 7.0.

Interaction	Association constant (M^{-1})	
	pH = 5.0	pH = 7.0
STI-CT	3.2×10^5	1.0×10^7
STI-Sepharose-CT	4.0×10^4	1.1×10^6

irradiated with γ-rays from a ^{60}Co source. Dose rates were determined by ferrous sulphate dosimetri [8]. The esterase activity of CT was measured using the potentiometric method introduced by Schwert et al. [9] To 10 ml substrate solution of pH = 7.00 (0.02 M N-Acetyl-l-Tyrosine-Ethyl-Ester, 0.2 M NaCl, 5 % (v/v) MeOH, and as buffering components, KH_2PO_4-Na_2HPO_4, of ionic strength 0.0025) in a thermostated (25.0°C) titration vessel of a Radiometer pH-Stat, 0.1 ml of enzyme solution was added with a calibrated Carlsberg pipette. In the pH-Stat, the pH was kept constant at, 7.00 ± 0.02, by addition of NaOH. From the rate of addition of base, v, the activity was determined as, v/c, where c is enzyme content. CT-catalyzed hydrolyses of esters can be represented by the following scheme [10]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow[+P_1]{k_3} E + P_2$$

where E and S represent enzyme and substrate respectively; P_1 (alcohol) and P_2 (acid), products. The relative activity is defined as, a'/a, where the prime indicates a modified enzyme. The catalytic constant, k_{cat}, is defined as, $k_2k_3/(k_2+k_3)$. When the apparent Michaelis-Menten constant, $K_M = (k_{-1}k_3 + k_2k_3)/(k_3k_1 + k_1k_2)$, is much smaller than the substrate content, the relative activity is approximately equal to

k'_{cat}/k_{cat}, the ratio of the catalytic constant of modified enzyme to the corresponding value for native CT.

Fig. 1 gives elution diagrams for native, heat- and radiation-treated enzyme samples. The protein content in the fractions is given by the absorbancy at 280 nm, A_{280}. The change in molar absorptivity was found to be negligible when 90 % of the enzyme sample had been inactivated by heat or radiation. The enzyme content in radiation-modified CT can therefore be approximated by A_{280}/ε, where ε is the molar absorptivity at 280 nm for native CT, 4.9×10^4 M^{-1} cm^{-1} [11]. The first peak in each diagram, with elution volume $\sim$ total column volume, consists of inactive products of low relative activity. The second peak in each diagram consists of native enzyme and products of high relative activity. These peaks are unsymmetric, indicating that the adsorption is governed by a non-linear adsorption isotherm. The fractions of higher relative activity are not well resolved, probably due to the following factors: (i) within the STI-Sepharose gel the adsorbent sites are not identical but there is a distribution of K_i-values; (ii) the radiation modification of CT gives products that cover the whole range of K_i-values $< K_i$ for native CT. These effects give rise to the observed overlapping of zones with different relative activities. But the observed increase in relative activity with elution volume give that K_i and thus K_{D_i} increases with relative activity. This is expected as K_i for a competitive inhibitor and the activity should be controlled by the same groups of the enzyme.

In the heat treatment all molecules should have been unfolded[12]. The elution diagram (Fig. 1 B) gives that all active molecules have the same relative activity as native CT. They were also found to have the same value of the wavelength for maximum fluorescence emission, λ_{max} = 331 nm, a structural parameter[13]. Heat-treated CT was separated with respect to molar volume in columns (14 x 1000 nm) packed with Sephadex G-100, at the same pH as the heat-treatment, pH = 3 and 7. The

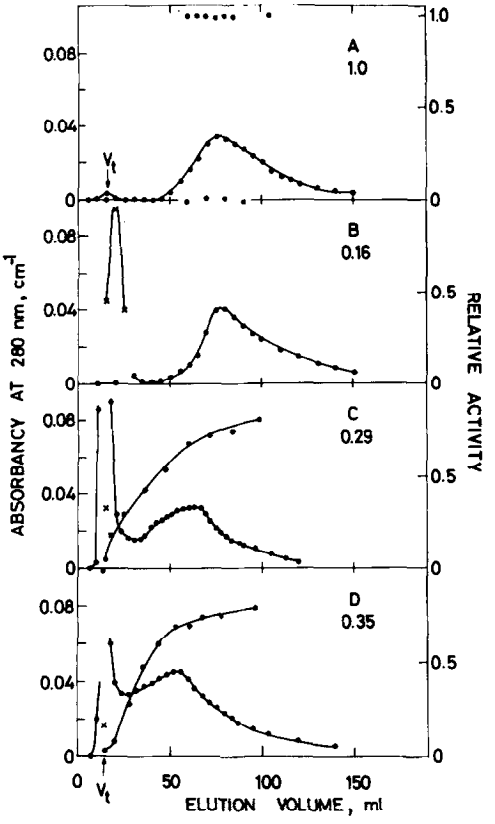

Figure 1. Elution diagrams for separations of native and modified CT in a STI-Sepharose column (14 x 65 mm). The elutions were performed at pH = 5.0 (HAc-NaOH, I = 0.05 total ionic strength adjusted to 0.25 with 0.2 NaCl), 22 ± 2 °C. The relative activity of the sample prior to separation is given in each diagram. The total capacity of the column was 20 mg native CT. Flow rate = 10 ml/hr.

●, absorbancy at 280 nm; x, absorbancy at 280 nm times 0.1; ○, relative activity; V_t, total column volume.

	sample	mg protein in sample
A	native CT	1.1
B	4×10^{-4} M CT kept 25 min at 50°C, pH = 3.0	6.2
C	4×10^{-4} M irradiated at pH = 7.0, dose 1.9 Mrad	1.8
D	CT irradiated dry, dose 14 Mrad	2.1

total ionic strength of the eluting buffer was 0.25. Only monomers,

indistinguishible from native CT, aggregates (at pH = 3) and autolysis products (at pH = 7) were found. This indicates that there exists only one stable folded conformation of this protein. Then non-covalent changes in monomeric CT cannot give stable monomeric products that differ from native CT.

In irradiated CT (Fig. 1 C and 1 D) a considerable amount of products with relative activities between 0 and 1 are formed. Fractions of relative activity > 0.5 were separated with respect to molar volume in columns (14x1000 mm) packed with Sephadex G-100 at pH = 7 (total ionic strength of eluting buffer 0.25). They had approximately the same elution volume as native CT. This indicates that all products with relative activities > 0.5 are monomers. As follows from the above results, these products must have undergone covalent changes (chemical modifications). Modifications that change the catalytic constant also give changes in K_M and the wavelength for maximum fluorescence emission (Table II). The results show that all permanent radiation-induced changes in monomeric CT are caused by chemical modifications that are responsible for observed changes in activity and the conformation parameter, λ_{max}. From the activity

Table II

Relative activity, apparent Michaelis-Menten constant, K_M, and wavelength of maximum fluorescence emission, λ_{max}, in fractions of modified CT separated with STI-Sepharose. Relative activity and K_M determinded at conditions given in text, λ_{max} was determined at pH = 5.0, same buffer as in Fig. 1.

Relative activity	K_M (mM)	λ_{max} (nm)
1.0 (native CT	3.7	331
0.85	4.0	332
0.71	6.2	333
0.52	8.7	334

distributions in Fig. 1 C and 1 D follows that gradual inactivation, formation of products with activities > 0, is of considerable importance in direct and indirect radiation inactivation of native CT.

This method to determine activity distributions in enzyme samples before and after modification, may be useful to study whether the native conformation is uniquely defined by the primary structure in other monomeric proteins.

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