Deacylation kinetics of γ-chymotrypsin in solution and in the crystal

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Deacylation kinetics of γ-chymotrypsin in solution and in the crystal

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The rate of catalyzed hydrolysis of the acyl enzyme analogs indolacryloyl-γ-chymotrypsin and furylacryloyl-γ-chymotrypsin in the crystal has been measured by single crystal microspectrophotometry and compared with the rate of catalyzed hydrolysis of acyl-γ-chymotrypsin in solution and acyl-α-chymotrypsin both in solution and in the crystal. The maximal deacylation rate is the same for both species and independent of the physical state. However, the pH dependence of the deacylation rate of crystalline acyl-γ-chymotrypsin shows a 0.9 unit shift in the pK of the catalytic system which is unique and probably consequent to specific lattice interactions.

Chymotrypsin *Serine protease* *Enzyme crystal* *Microspectrophotometry*

1. INTRODUCTION

The comparison of the refined structures of γ-chymotrypsin at 1.9 Å resolution [1], α-chymotrypsin at 1.68 Å resolution [2] and the dimer of α-chymotrypsin at 1.67 Å resolution [3] has revealed the existence of several differences among the various crystalline forms of the enzyme. A major difference within the active site region is the distance between Oγ of Ser-195, and Nε2 of His-57 which is compatible with the formation of a hydrogen bond in α-chymotrypsin [2], as well as in other homologous serine proteases [4–9], but is hardly compatible with it in γ-chymotrypsin [1] or in the dimer of α-chymotrypsin [3].

α- and γ-chymotrypsin are chemically and functionally identical molecules, although distinguishable by their crystallization properties, the only known distinction in solution being the dimerization properties of the enzyme at acidic pH [10].

In the crystal, it is not possible to measure and compare the rates of reaction with substrates of the two enzyme forms, since diffusion of relatively large molecules through the aqueous channels could be the common rate limiting process and obscure the possible variances in the reaction rates. Therefore, it cannot be directly established whether the observed structural differences, some of which might be induced by specific lattice interactions, have a functional consequence.

To enquire whether crystalline γ-chymotrypsin might represent a somewhat altered structural and functional state of the enzyme, we adopted the criterion used by Rossi and Bernhard [11] to establish the full activity of α-chymotrypsin crystallized according to Sigler and Blow [12]. We therefore prepared the chromophoric acyl enzyme derivatives, indolacryloyl-γ-chymotrypsin and furylacryloyl-γ-chymotrypsin, and measured the rates of catalyzed deacylation within single enzyme crystals.

Since the crystals of γ-chymotrypsin are stable over and beyond the full range of catalytic interest, from pH 2 to pH 11 [1], it was possible to analyze the pH–rate profile over a much wider range than in the case of α-chymotrypsin.

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2. MATERIALS AND METHODS

Tetragonal crystals of γ-chymotrypsin (space group $P4_22_12$) identical to those used for the crystallographic studies [1], were a generous gift of Dr B.W. Matthews. Crystals with identical properties were prepared in our laboratory from unbuffered solutions containing 10 mg/ml protein (Worthington), ammonium sulfate (50% saturation), at room temperature. α-Chymotrypsin was crystallized according to Sigler and Blow [12]. Crystals of α- and γ-chymotrypsin were stored at 4°C, in a 60% saturated ammonium sulfate solution, pH 4.0 and 5.5, respectively.

The acylating reagents *N*-β-(3-indol)acryloylimidazole and *N*-β-(2-furyl)acryloylimidazole were prepared as described [13]. The reason for choosing these reagents was the interest to examine at least two chromophoric acyl enzyme derivatives that in solution exhibit different p*K* and maximal rate of deacylation [14]. For acylation, crystals were soaked for 30 min in a solution obtained by adding microliter quantities of a saturated solution of the acylating reagent in diethylene dioxide to 5 ml of 60% saturated ammonium sulfate, 50 mM citrate, pH 4.0. The final concentration of either acylating reagent was 5×10^{-5} M. At this pH the acyl enzyme intermediate was stable for several days [11]. Individual intact crystals were selected, washed and resuspended in 60% saturated ammonium sulfate solution at the desired pH. As verified in the previous work [11], pH equilibration between the new medium and the solvent inside the crystal occurred before starting spectrophotometric measurements.

Single crystal spectra in polarized light were recorded by using a Zeiss UV-visible MPM 03 microspectrophotometer.

Deacylation rates of *O*-β-(3-indol)acryloyl-Ser-195-γ-chymotrypsin at different pH were measured at 18°C by monitoring the pseudo first order decrease of the absorbance at $\lambda = 360$ nm, the maximum of the absorbance of the catalytically active acyl enzyme [13]. The indolacrylate product does not appreciably absorb at this wavelength [15]. Deacylation rates of *O*-β-(2-furyl)acryloyl-Ser-195-γ-chymotrypsin were similarly followed at $\lambda = 320$ nm at 25°C [13].

Control experiments were carried out by measuring spectra and deacylation rates of both acyl-α- and acyl-γ-chymotrypsin in solution, in the presence of 45% saturated ammonium sulfate, a salt concentration still compatible with the soluble state of the enzyme. The earlier measurements on α-chymotrypsin crystals [11] were also repeated and fully confirmed.

3. RESULTS AND DISCUSSION

The polarized absorption spectra of single crystals of γ-chymotrypsin exposed to either *O*-β-(3-indol)acryloylimidazole and *O*-β-(2-furyl)acryloylimidazole exhibit a maximum at $\lambda = 360$ and $\lambda = 320$ nm, respectively (fig.1), characteristic for either acyl enzyme derivative in the catalytically active form in solution [13]. This finding implies that the active site structure of γ-chymotrypsin in the crystal is catalytically competent. In both cases, the electric dipole transition moment direction is almost exactly parallel to the C_4 axis of the crystal.

The pH dependence of the rate of catalyzed hydrolysis of indolacryloyl-γ-chymotrypsin is reported in fig.2A. In solution, at every pH value, the rate of hydrolysis of indolacryloyl-γ-chymotrypsin is identical with that reported for indolacryloyl-α-chymotrypsin [14]. The pH-rate profile is a single sigmoid, characterized by $pK = 7.6$ and $k_{max} = 0.070\ min^{-1}$ at 18°C. The rates of catalyzed hydrolysis of the acyl enzyme in solution were found to be essentially independent of the presence of ammonium sulfate.

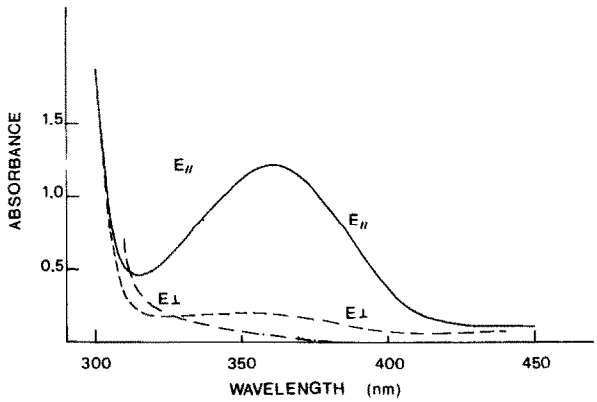

Fig.1. Polarized spectra of single crystals of indolacryloyl-γ-chymotrypsin (——,– – –) and furylacryloyl-γ-chymotrypsin (· · ·,– · – · –). The orientation of the electric dipole transition moment (*E*) is relative to the C_4 axis of the crystal.

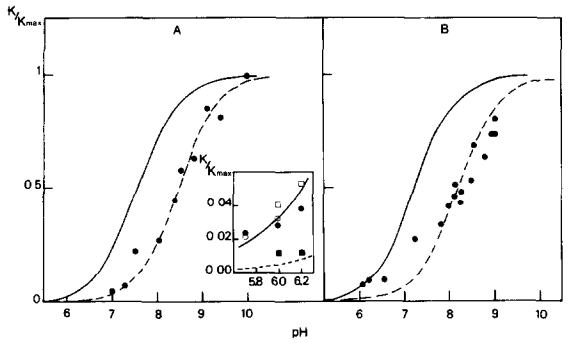

Fig.2.(A) pH dependence of the deacylation rate of both indolacryloyl-α- and γ-chymotrypsin in solution (solid line; k_{max} = 0.070 min^{-1}, pK = 7.6) and of crystalline indolacryloyl-γ-chymotrypsin (●) at 18°C. The dashed line is the theoretical sigmoid calculated for k_{max} = 0.070 min^{-1} and pK = 8.5. (Inset) Comparison on expanded scale of the deacylation rates of indolacryloyl-α-chymotrypsin (○,●) and indolacryloyl-γ-chymotrypsin (□,■) in solution (○,□) and in the crystalline (●,■) state at 25°C. Solid and dashed lines are the theoretical sigmoids calculated for k_{max} = 0.113 min^{-1} and pK 7.6 and 8.5, respectively. (B) pH dependence of the deacylation rate of both furylacryloyl-α- and γ-chymotrypsin in solution (solid line; k_{max} = 0.143 min^{-1}, pK = 7.3) and of crystalline furylacryloyl-γ-chymotrypsin (●) at 25°C. The dashed line is the theoretical sigmoid calculated for k_{max} = 0.143 min^{-1} and pK = 8.2.

In the case of crystalline indolacryloyl-α-chymotrypsin, the structure of which has been determined [16], the rates of hydrolysis are the same as in solution, in the pH range (5.7–6.2) where crystals are relatively stable (fig.2A, inset). In the case of crystalline indolacryloyl-γ-chymotrypsin, the maximal rate of hydrolysis is identical with that measured in solution, but the apparent pK of the catalytic system is raised by 0.9 pH units (fig.2A). This result was confirmed by the comparison of the pH dependence of the rate of hydrolysis of the furylacryloyl derivative of γ-chymotrypsin in solution and in the crystal (fig.2B).

Since these data reveal the occurrence of a lattice-induced change in the ionization properties of the acyl enzyme active site, it might be inferred that the increased distance between Oγ of Ser-195 and Nϵ2 of His-57 in native γ-chymotrypsin with respect to α-chymotrypsin, which is incompatible with the formation of the hydrogen bond and is unique to γ-chymotrypsin among several homologous serine proteases in the crystal [2,4–9], is also consequent to crystal packing.

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