CHANGE IN THE CONFORMATION OF δ-CHYMOTRYPSIN UPON BINDING A SPECIFIC SUBSTRATE AT HIGH pH.

Jean-Renaud GAREL*
Laboratoire d'Enzymologie, Institut de Biochimie, Université de Paris-Sud, 91405-Orsay, France

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1. Introduction

It is well established that δ-CT* can exist under two main conformations, the equilibrium between which depends upon the ionization state of the α-amino group of Ile 16 [1–5]; the first one, the 'active' or 'neutral pH' conformation predominates at neutral pH where the Ile 16 is protonated and is able to bind specific ligands, substrates or inhibitors, whereas the other one, the 'inactive' or 'high pH' conformation prevails at alkaline pH where the Ile 16 is deprotonated and seems to be less capable of binding [4–7]. It has been observed that the non-covalent binding of an inhibitor to δ-CT displaces the equilibrium between these conformations, and apparently results in a ligand induced conversion from the inactive to the active conformation [5,8,10]. The same conversion is thought to take place when a substrate binds to the enzyme, and thus to explain why δ-CT is active at high pH, although with a decreased affinity [5,7]. The few investigations performed with real substrates have supported this view; by potentiometric techniques, McConn et al. [11] have measured the change in the pK of Ile 16 upon binding of ATrA to δ-CT, and it has been previously shown that the fluorescence of the protein varied upon binding an ester substrate [10]. We wish to present results on the binding of ATrA to δ-CT at high pH which show that; 1) there is a substrate promoted isomerization of δ-CT from its inactive to its active conformation, and 2) there is a good correlation between the pK shift of Ile 16 induced by ATrA and the K_m change for this substrate when the pH is raised from neutral to alkaline.

2. Materials and methods

δ-CT was prepared from three times crystallized chymotrypsinogen (Worthington, U.S.A.) as described previously [10]. Protein concentrations were determined spectrophotometrically using a molar absorbance of 5×10^4 at 280 nm [12]. ATrA and ATEE were obtained from Cyclo (U.S.A.); all other reagents were from Merck (Germany) or Prolabo (France).

All experiments were carried out at 15°C, in 20 mM buffer (pyrophosphate, tris or carbonate) and 150 mM KCl.

Optical rotation measurements were performed with a Perkin-Elmer 141 polarimeter at 365 nm using a 10 cm optical pathway. Since both the enzyme and its substrate contribute to the observed optical rotation, we used an experimental procedure which assured identical δ-CT and ATrA concentrations throughout a series of measurements; a stock solution of δ-CT (0.8 to 1.2 mg/ml) and ATrA (0 to 15 mM) in 150 mM KCl was kept at pH 3, 0°C; a 2 ml sample was taken and allowed to equilibrate at 15°C for a few minutes; the pH of this sample was then raised by the addition of 20 μl of a 2 M buffer solution (tris or carbonate) and the optical rotation was measured; the actual pH of the sample was determined

* Present address: Service de Biochimie Cellulaire, Institut Pasteur, 25 Rue du Docteur Roux, 75015-Paris, France.
* *Abbreviations:* δ-CT: δ-chymotrypsin. ATrA: *N*-acetyl-L-tryptophanamide. ATEE: *N*-acetyl-L-tyrosine ethyl ester.

at 15°C just after the polarimetric run. The catalytic rate constant of δ-CT towards ATrA depends on a pK of 7 and is equal to 7×10^{-2} sec^{-1} at pH 8, 25°C [6]; at pH 3, 0°C, this constant is less than 10^{-5} sec^{-1}, and therefore only a negligible amount of ATrA will be hydrolyzed during the longest time the same stock solution was used, about one hour.

The time course of the δ-CT catalyzed hydrolysis of ATEE has been followed according to a previously described procedure [10]. δ-CT (1 to 2 mg/ml) in the presence of ATrA (0 to 15 mM) was incubated at various pH values at 15°C in 150 mM KCl; a 5 to 20 μl sample was rapidly diluted in 5 ml of a solution containing 10 mM ATEE, 20 mM pyrophosphate, 150 mM KCl, at pH 8.2, 15°C, and the amount of protons released was recorded with time.

The activity of δ-CT towards ATrA was measured by following the rate of release of ammonia [13] in conditions identical to those used in polarimetric measurements.

3. Results

At pH 7.5, the optical rotation of a mixture of δ-CT and ATrA is the sum of the optical rotation of the separated reactants (fig.1), showing that the binding of ATrA does not cause any major change in the 'neutral pH' conformation of the enzyme. This additivity is no longer observed at high pH. As the optical rotation of ATrA is pH independent, the deviations from additivity have to come from the enzyme (fig.1). Fig.2 shows the pH dependence of the optical rotation changes of δ-CT in the presence of various ATrA concentrations. In the absence of substrate, δ-CT undergoes a pH dependent transition with an apparent pK of 9 ± 0.05, in agreement with other reported values [9,11,14]. In the presence of increasing concentrations of ATrA, the amplitude of this transition is decreased and the half transition pH is shifted towards higher pH values (fig.2). At each pH value, the extrapolation to saturation in ATrA was

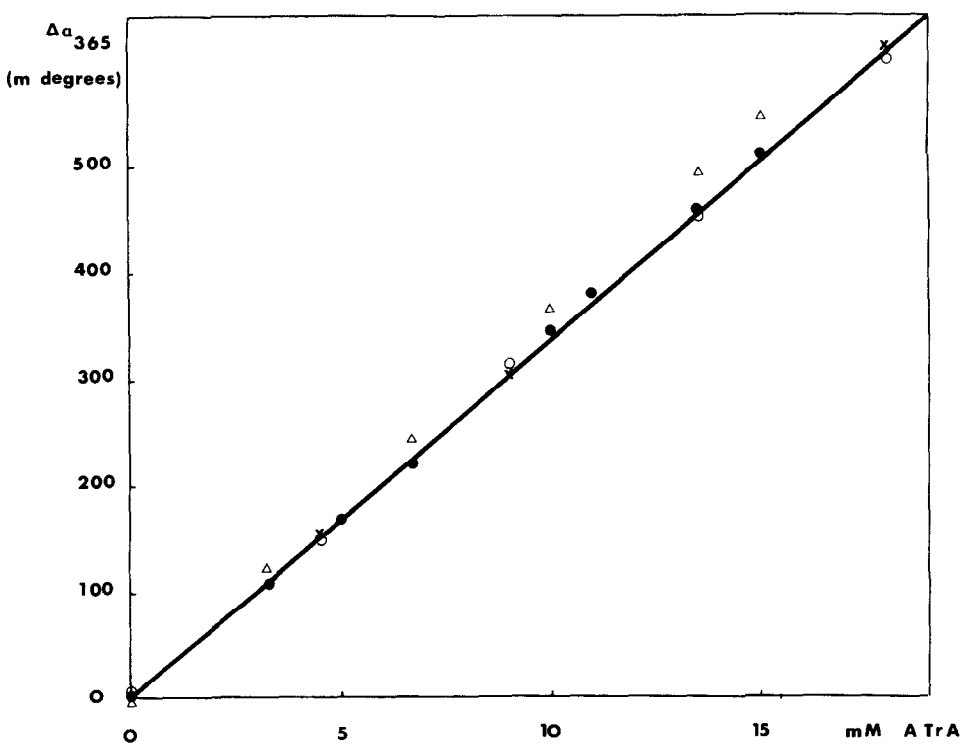

Fig.1. Changes in optical rotation observed at 365 nm upon addition of increasing concentrations of ATrA to solutions containing: (○) buffer at pH 7.5; (×) buffer at pH 10.4; (●) -CT (0.5 to 1.5 mg/ml) at pH 7.5; (△) -CT (1.5 mg/ml) at pH 10.4; other conditions as described in methods section. Optical pathway 10 cm.

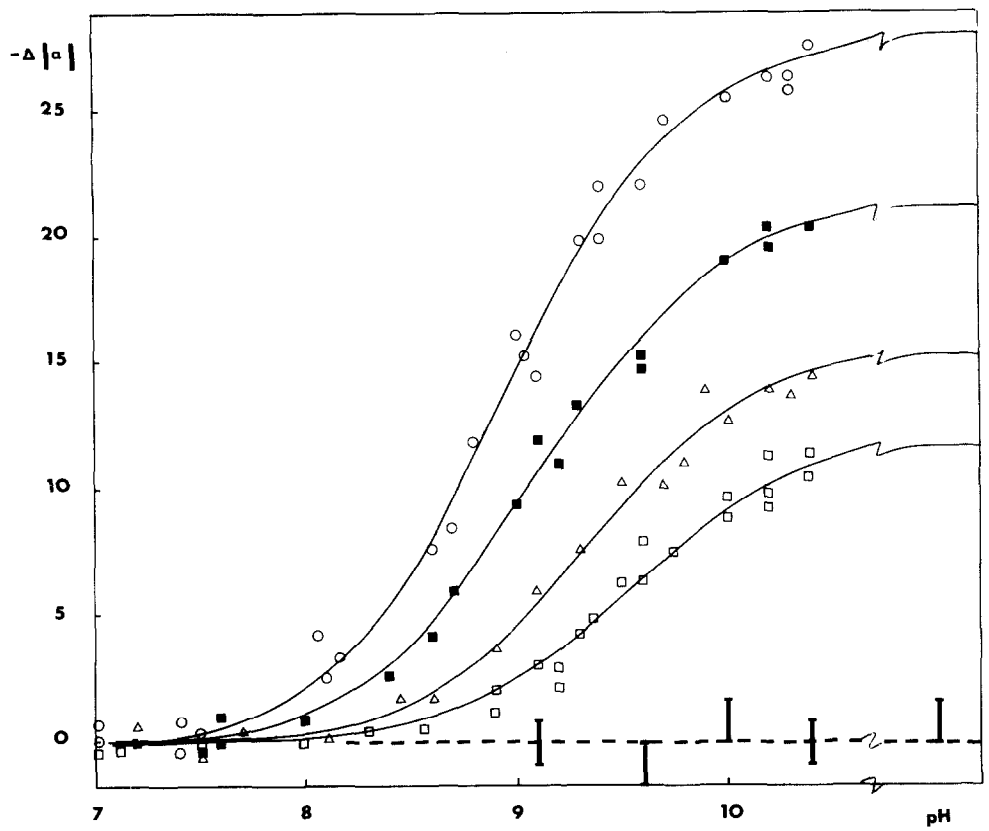

Fig.2. pH dependence of the changes in specific optical rotation at 365 nm of δ-CT in the presence of: (○) no ATrA: (X) 3.3 mM ATrA; (△) 6.7 mM ATrA; (□) 13.5 mM ATrA.
The vertical bars (I) indicate the extrapolation of these changes to saturation in ATrA as determined from plots similar to that given in fig.3.

achieved by a double reciprocal plot* (as shown in fig.3 for the high pH plateau values of the optical rotation); such a plot gives the values of the optical rotation of the δ-CT-ATrA complex and of the apparent binding constant of ATrA to δ-CT at this pH. From fig.3 and similar data, it was found that the optical rotation of the d-CT-ATrA complex was pH independent with a value close to that of free δ-CT at neutral pH (fig.2).

It has been previously shown that, beside their difference in optical rotation, the 'neutral pH' and 'high pH' conformations of δ-CT exhibit different kinetic behaviours in the hydrolysis of ATEE: the 'neutral pH' form shows immediately its catalytic activity, whereas the 'high pH' form needs a lag period before reaching the same activity [10]. In the presence of increasing ATrA concentrations, this lag phase shown by the 'high pH' form of δ-CT is progressively suppressed, indicating that binding of ATrA converts the enzyme into an immediately active species. Fig. 4 shows the determination of the apparent binding constant of ATrA to δ-CT from measurements of the lag phase (see [10,15] for a complete example of treatment of such data).

The pH dependence of the activity of δ-CT towards ATrA has been investigated with results very similar to those already reported: the catalytic

* This treatment of the data is very similar to that of analogous results obtained previously with acetylated δ-CT and indole [8].

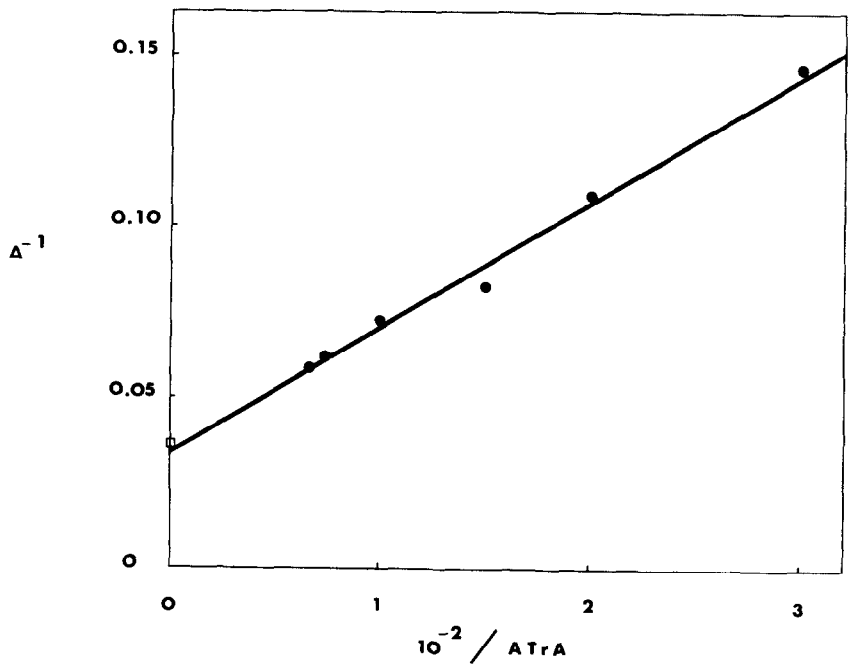

Fig.3. Double reciprocal plot of the changes in specific optical rotation of δ-CT in the presence of ATrA, corresponding to the high pH plateaus of the curves of fig.2. △ is the difference between the values of the optical rotation of δ-CT in the absence of ATrA and in the presence of a given concentration of ATrA. The symbol (□) corresponds to the total change observed for δ-CT in the absence of ATrA upon raising the pH; note the similarity between this change and the one obtained by extrapolation in ATrA.

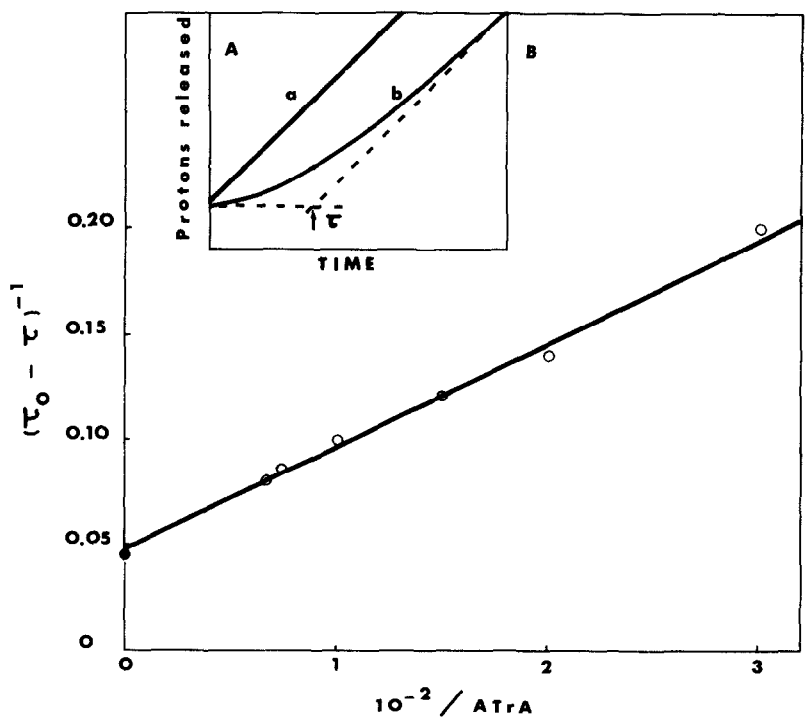

Fig.4

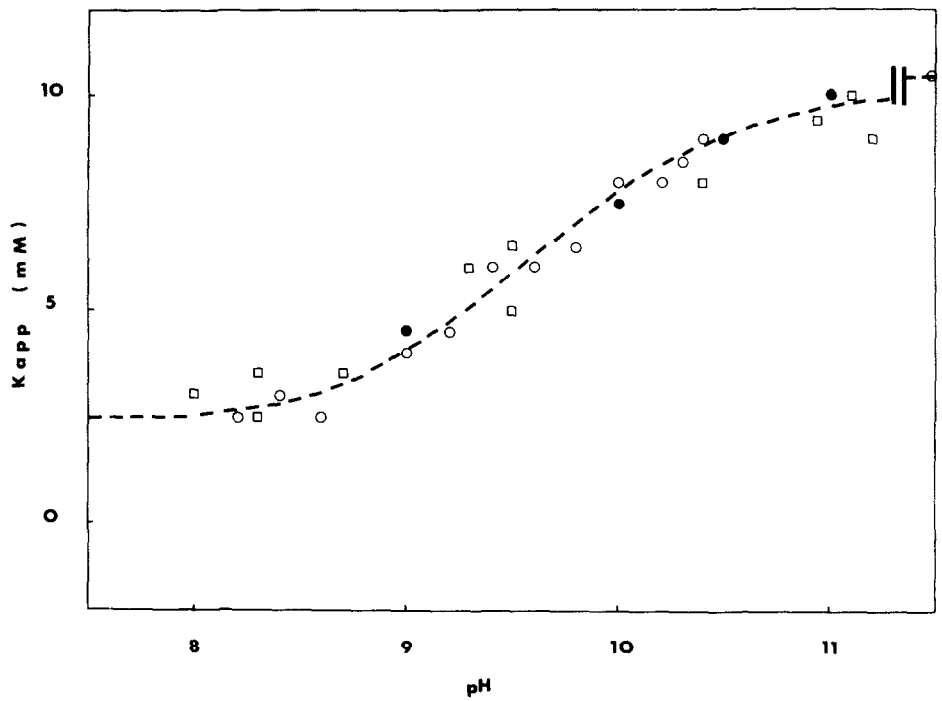

Fig.5. pH dependence of the apparent binding constant K_{app} of ATrA to δ-CT as measured by: (□) the Michaelis constant in the hydrolysis of ATrA by δ-CT; (•) the inhibition of the lag phase in the hydrolysis of ATEE (fig.4). (○) the changes in optical rotation (figs.2 and 3); the high pH point is obtained from fig.3.

constant remains constant with pH while the Michaëlis constant increases [6]. Fig.5 gives the pH dependence of the apparent binding constant of ATrA to δ-CT as measured by changes in the enzyme optical rotation, suppression of the lag phase in the hydrolysis of ATEE, and Michaelis constant of the hydrolysis of ATrA. These three methods give the same results for the value of the binding constant and its pH dependence.

4. Discussion

The above results show that the binding of ATrA to δ-CT converts the 'high pH' form into a conformation that; 1) has a pH independent optical rotation, the value of which is similar to that of the 'neutral pH' form, and 2) does not need a lag period to express its enzymic activity (figs. 3 and 4). Both of these properties indicate that upon binding ATrA, the enzyme is locked in a conformation which very much resembles that seen at neutral pH, and which is no longer sensitive to pH, up to 10.5 (fig.2). By proton uptake measurements, McConn et al. [11] have shown that the apparent pK of the group involved in the conformational equilibrium, i.e. the pK of the α-amino group of Ile 16, was shifted from about 9 in the free enzyme to about 9.6 in the δ-CT-ATrA

Fig.4. (A) Time course of the hydrolysis of ATEE by δ-CT when the enzyme is initially at neutral pH (curve a) and at high pH (curve b); the lag time τ is defined by the intersection of the steady state line with the base line (see [10,15] for the analysis of such curves). Experimental conditions given under Methods. (B) Double reciprocal plot showing the suppression of the lag phase upon binding of ATrA at pH 11; τ_0 and τ are the lag times in the absence and the presence of ATrA respectively. The symbol (•) corresponds to the reciprocal of τ_0; note that τ has a value close to zero at saturation in ATrA $(\tau_0-\tau)^{-1}$ is close to τ_0^{-1}).

complex. Fig.5 shows that going from neutral to high pH makes the binding constant of ATrA to δ-CT increase from 2.5 to 10 mM. If the dissociation of the Ile 16 proton makes the binding of ATrA four times more difficult, then the binding of ATrA should make the binding of the Ile 16 proton four times easier, i.e. a pK shift from 8.95 in the absence of ATrA (fig.2) to 9.55 (= 8.95 + log 4) at saturation in ATrA, which is observed. There is then a quantitative agreement between the results of studies of the binding of ATrA to δ-CT at high pH by different methods. This agreement allows to make the following conclusions:

i) binding of ATrA causes the enzyme to remain in an 'active-like' conformation (as seen from both optical rotation and enzymic activity) even at pH values where the α-amino group of Ile 16 is no longer protonated;

ii) binding of ATrA causes the apparent pK of Ile 16 to shift from about 9 in the free enzyme (where this pK is linked to a conformational equilibrium) to about 9.6 in the δ-CT-ATrA complex (where ionization of Ile 16 is no longer accompanied by a detectable conformational change);

iii) the conversion from the 'high pH' form to this 'active-like' one, upon binding ATrA, results in a decrease in the affinity for ATrA which quantitatively corresponds to the decrease in affinity observed by classical steady state kinetics.

All presently available informations seem to indicate that this ligand-promoted isomerization arises from the lack of binding ability of the 'high pH' form and corresponds to a shift of the conformational equilibrium in favor of the better binding conformation [9,10].

However when the binding of a proton to isoleucine 16 was studied either through indole binding to the specificity site [8] or through the kinetics of conformational changes after pH jumps, a pK shift from 9 to 10.5 was observed for this group instead of the shift presently found from 9 to 9.6. The pK of 10.5 is that of Ile 16 in the active form of the enzyme [8,16] and has independently been determined by proflavine binding studies [9]. A shift of 1.5 pH unit corresponds to a 30-fold change in apparent affinity; this is quite different from the 4-fold change presently observed when ATrA is used as a specific ligand. Therefore a 7 to 8-fold difference (30/4) appears in the affinity of protons to Ile 16 in the active form of δ-CT, depending on the ligand used to study this affinity. If one admits that the pK of 10.5 found by totally different methods, either for the free enzyme or for the indole or proflavine bound one, is the correct value, one has to admit that the pK 9.6 found by McConn et al. [11] and by ourselves in presence of ATrA results from some unknown perturbation. This cannot be linked to conformational differences of the active protein brought about by ATrA if the optical rotation of the enzyme is taken as a structural probe. Furthermore since there is a very good parallel between the △ pK of Ile 16 and the K_m change of ATrA, this apparent △ pK is likely devoid of gross experimental errors. We conclude that a kinetic step, different from the formation of an acyl enzyme, should be searched to explain the anomalous data presently or already [11] observed in the hydrolysis of ATrA at high pH.

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