Johnson, F. H., and Schlegel, F. M. (1948), *J. Cell. Comp. Physiol. 31,* 421.

Kauzmann, W. (1959), *Advan. Protein Chem. 14,* 1.

Kendrew, J. C. (1962), *Brookhaven Symp. Biol. 15,* 216.

Kohnstam, G. (1970), *Progr. React. Kinet. 5,* 335.

La Gow, J., and Parkhurst, L. J. (1972), *Biochemistry 11,* 4520.

Laidler, K. J. (1958), The Chemical Kinetics of Enzyme Action, London, Oxford University Press, p 210.

Le Noble, W. J. (1965), *Progr. Phys. Org. Chem. 5,* 207.

McCray, J. (1972), *Biochem. Biophys. Res. Commun. 47,* 187.

Nobbs, C. L., Watson, H. C., and Kendrew, J. C. (1966), *Nature (London) 209,* 339.

Noble, R. W., Brunori, M., Wyman, J., and Antonini, E. (1967), *Biochemistry 6,* 1216.

Olson, J. S., and Gibson, Q. H. (1973), *J. Biol. Chem. 248,* 1623.

Perutz, M. F. (1970), *Nature (London) 228,* 726.

Rasper, J., and Kauzmann, W. (1962), *J. Amer. Chem. Soc. 84,* 1771.

Schellmann, J. A. (1955), *C. R. Trav. Lab. Carlsberg 29,* 223.

Schmelzer, U., Steiner, R., Mayer, A., Nedetzka, T., and Fasold, H. (1972), *Eur. J. Biochem. 25,* 491.

Suzuki, K., Taniguchi, Y., and Izui, K. (1972), *J. Biochem. 71,* 901.

Whalley, E. (1964), *Advan. Phys. Org. Chem. 2,* 93.

Wittenberg, B. A., Brunori, M., Antonini, E., Wittenberg, J. B., and Wyman, J. (1965), *Arch. Biochem. Biophys. 111,* 576.

Zipp, A., Ogunmola, G., Neuman, R. C., and Kauzmann, W. (1972), *J. Amer. Chem. Soc. 94,* 2541.

The Acidic Transition of δ-Chymotrypsin†

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ABSTRACT: The behaviors of chymotrypsinogen and δ-chymotrypsin have been studied at acidic pH by optical rotation, absorption, and fluorescence measurements; both proteins show pH-dependent changes. Kinetic experiments using either absorption or fluorescence and a pH jump method have evidenced a slow process which takes place in the enzyme and not in its zymogen; this slow process is controlled by the ionization of a group with a pK of 3, and involves large fluorescence changes. By correcting the changes observed in δ-chymotrypsin at equilibrium from those observed in the zymogen, one may evaluate the variations specifically linked to the ionization of the group of pK ~ 3; these variations appear to be very similar to those linked to the ionization of the α-amino group of Ile-16. This result and all the available information make likely the assignment of the pK of 3 to the β-carboxyl group of Asp-194. A more detailed kinetic investigation using fluorescence measurements suggests that δ-chymotrypsin exists under two main conformations, in which this residue has very different pK's. The relevance of the conformational importance of this group in the enzyme to the activation process of the zymogen is also discussed.

One of the critical features of the conformation of chymotrypsin, as revealed by X-ray crystallography, is the existence of an electrostatic interaction between the α-amino group of Ile-16 and the β-carboxyl group of Asp-194; this salt bridge is buried inside the protein molecule and shielded from solvent (Matthews *et al.*, 1967; Sigler *et al.*, 1968). In chymotrypsinogen the α-amino group of Ile-16 forms a peptide bond with Arg-15 and cannot interact with Asp-194. The formation of the Ile-16–Asp-194 interaction appears to be a major event in the activation process, as has been summarized by Hess (1971): "The key which unlocks the inactive conformation is the α-amino group of Ile-16. When this α-amino group is liberated in the conversion of chymotrypsinogen to chymotrypsin at neutral pH, it acquires a positive charge. This positively charged group induces ion pair formation with Asp-194 and the resulting movement of the peptide chains establishes a specific substrate binding site." This remark emphasizes the close connection which exists between the activation process of chymotrypsinogen on one hand, and the role of the Ile-16–Asp-194 interaction in chymotrypsin on the other hand. When lacking the Ile-16–Asp-194 salt bridge (*i.e.*, when the charge of Ile-16 is suppressed by either deprotonation or chemical modification), the enzyme is in a conformation which looks like that of the zymogen, as seen from its circular dichroism spectrum, its optical rotatory dispersion, and its activity toward specific substrates[1] (Oppenheimer *et al.*, 1966; Mc Conn *et al.*, 1969; Ghelis, 1971; Hess *et al.*, 1970; Garel and Labouesse, 1970, 1973; Hess, 1971). We have previously proposed a two-step model for the activation process of chymotrypsinogen: the trypsin catalyzed cleavage of the Arg-15–Ile-16 peptide bond would be an irreversible first step, whereas the second and reversible step

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[1] The activity of the conformation lacking the Ile-16–Asp-194 salt bridge toward nonspecific substrates is still under controversy: we have found it capable of hydrolyzing *p*-nitrophenyl acetate (Ghelis *et al.*, 1970) whereas another report claims it could not (Fersht, 1973). In the case of the related enzyme trypsin, a derivative blocked on its N-terminal α-amino group is still able to hydrolyze *p*-nitrophenyl-*p'*-guanidinobenzoate, a pseudosubstrate, although it is inactive toward specific substrates (Robinson *et al.*, 1973).

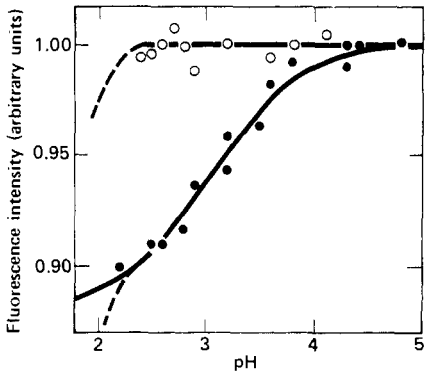

FIGURE 1: pH dependence of the fluorescence emitted at 335 nm by δ-chymotrypsin (●) and by chymotrypsinogen (○) when excited at 290 nm. The solid line for the enzyme is the ionization curve of a group of $pK = 3$. The dashed lines correspond to the sharp decrease in fluorescence observed below pH 2 and attributed to the protonation of indole rings. The fluorescence of both proteins has been taken as the same at pH 5.

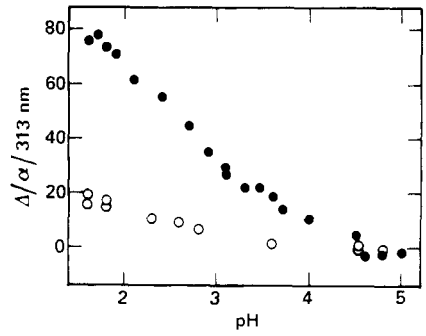

FIGURE 2: pH dependence of the specific optical rotation at 313 nm of δ-chymotrypsin (●) and chymotrypsinogen (○) at 15° in 150 mM KCl.

would be the conformational rearrangement controlled by the formation of the Ile-16–Asp-194 interaction (Ghélis *et al.*, 1970); this second step would therefore be identical with the transition undergone by chymotrypsin when brought from alkaline to neutral pH, which has already been studied in some detail.

It has been previously pointed out that the formation of the Ile-16–Asp-194 salt bridge in chymotrypsin should be achieved as well by the ionization of Asp-194, *i.e.*, by bringing the protein from acidic to neutral pH, as by the protonation of Ile-16 (Garel and Labouesse, 1970), and the aim of this work is to monitor and possibly characterize the conformational change linked to the changes in the ionization state of Asp-194. pH dependent conformational changes have already been reported for chymotrypsin at low pH (Rupley *et al.*, 1955; Parker and Lumry, 1963; Biltonen *et al.*, 1965a; Lazdunski *et al.*, 1970); these changes, however, cannot be directly related to Asp-194, since other groups also ionize at low pH and may contribute to the observed changes. In fact chymotrypsinogen, in which no Ile-16–Asp-194 interaction can exist, also shows pH dependent changes at low pH (Biltonen *et al.*, 1965b, 1971; Delaage *et al.*, 1968; Lazdunski *et al.*, 1970; Bellon and Delaage, 1970). It is neither possible to isolate the effect of Asp-194 ionization by chemically blocking all other carboxyl groups (as has been done in the case of Ile-16 (Ghelis *et al.*, 1967)), since this modification does not lead to a derivative with a single ionizable group, and may even alter the properties of the protein (Carraway *et al.*, 1969; Abita and Lazdunski, 1969). Another carboxyl group, that of the Asp-102 side chain, appears to be buried both in chymotrypsin (Blow *et al.*, 1969) and its zymogen (Freer *et al.*, 1970; Kraut, 1971), and it is not accessible to carboxyl group reagents (Fersht and Sperling, 1973). Some *a priori* features are expected from a conformational change specifically controlled by the ionization of Asp-194 and the concomitant formation of its interaction with Ile-16: (a) this change should be observed in chymotrypsin and not chymotrypsinogen; (b) this change should pertain to a single group, and therefore should give identical transition curves when measured by different parameters (Tanford, 1961); (c) the properties of this change should be consistent with those already known for the Ile-16–Asp-194 salt bridge from studies on the Ile-16 side.

This paper reports the results of a comparative study of the behaviors of δ-chymotrypsin and chymotrypsinogen at low pH, and shows that the enzyme undergoes a conformational change which is likely to be controlled by the β-carboxyl group of Asp-194, although the involvement of this residue has not been proven definitively.

Materials and Methods

Three times crystallized chymotrypsinogen and crystallized trypsin were obtained from Worthington. δ-Chymotrypsin was prepared by activation of the zymogen according to a previously described procedure (Garel and Labouesse, 1973). The enzyme obtained showed (i) 90–95% active site content when assayed with *p*-nitrophenyl acetate, (ii) a catalytic constant of 230 sec^{-1} toward *N*-acetyl-L-tyrosine ethyl ester at pH 8, 25°, and (iii) less than 0.1 N-terminal Ala residue per mole of protein as measured by the dansylation technique (Gros and Labouesse, 1969). Protein concentrations were determined spectrophotometrically using a molar absorbance of 5×10^4 at 280 nm for both chymotrypsinogen and δ-chymotrypsin (Dixon and Neurath, 1957).

All experiments were carried out in thermostated cells at 15°, where no thermal denaturation occurs (Biltonen and Lumry, 1969), and under conditions where the aggregation is negligible for both proteins (Miller *et al.*, 1971). Protein solutions were made in 150 mM KCl; 20 mM citrate buffer was used down to pH 2 and no buffer was used below this value. The pH of the solutions was measured at 15° at the end of each experiment.

Optical rotation measurements were performed at 313 nm with a Perkin-Elmer 141 spectropolarimeter, using a 10-cm optical pathway with protein concentrations ranging from 1 to 1.5 mg/ml. Fluorescence measurements were carried out with a Farrand Mark 1 spectrofluorometer on protein solutions at about 0.15 mg/ml. Difference spectra were recorded with a Cary 15 spectrophotometer on protein solutions at a concentration close to 1 mg/ml. Kinetics of fluorescence and absorption changes were followed with a Durrum-Gibson stopped flow apparatus; the pH jump method previously described (Garel and Labouesse, 1971) was used here with solutions at protein concentrations ranging from 0.1 to 0.5 mg/ml. In absorption experiments, the monochromator slits were set at a width of 0.3 mm or less, so that the spectral dispersion never exceeded 2 nm (as determined from the manufacturer specifications).

Results

Conformational Changes in δ-Chymotrypsin and Chymotrypsinogen at Equilibrium. Figure 1 shows the pH dependence of the fluorescence emitted at 335 nm by δ-chymotrypsin and its zymogen, when excited at 290 nm. The fluorescence of chymotrypsinogen remains constant above pH 2, whereas that of δ-chymotrypsin increases by about 10% with pH and follows the ionization curve of a group of $pK \sim 3$. Below pH 2, the fluorescence of both proteins decreases similarly, which may be

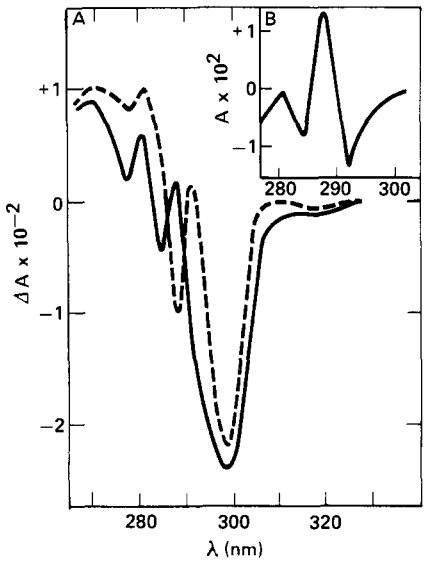

FIGURE 3: (A) Difference spectra of δ-chymotrypsin (—) and its zymogen (- - -) pH 1.9 *vs.* pH 4.3. The chymotrypsinogen spectrum is the same as that reported by Delaage *et al.* (1968). Protein concentration, 1 mg/ml. (B) Difference between the spectra of Figure 3A, δ-chymotrypsin *vs.* chymotrypsinogen.

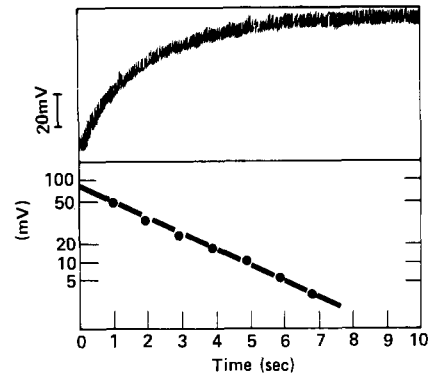

FIGURE 4: Oscilloscope tracing (top curve) of the fluorescence change observed upon a pH jump from pH 2 to pH 4.5 in δ-chymotrypsin. Final conditions: δ-chymotrypsin, 0.25 mg/ml; 150 mM KCl; 20 mM citrate buffer; pH 4.5; 15°. Total signal for the protein at pH 4.5, 800 mV. Semilogarithmic plot (bottom curve) of the trace given in the top curve.

due to the protonation of indole rings (Bridges and Williams, 1968).

The specific optical rotation at 313 nm of chymotrypsinogen is slightly pH dependent below pH 3, while that of δ-chymotrypsin shows much larger variations (Figure 2). The pH dependence of the changes in optical rotation of δ-chymotrypsin appears, however, to be different from that of the fluorescence changes.

Both chymotrypsin and its zymogen are known to exhibit pH-dependent absorption changes (Lazdunski *et al.*, 1970; Bellon and Delaage, 1970). Figure 3A gives the difference spectra obtained for both proteins upon lowering the pH from 4.3 to 1.9. The main feature of these spectra is a large band centered around 300 nm; however, this band has about the same amplitude for δ-chymotrypsin and chymotrypsinogen. Nevertheless, spectral differences can be seen in the 280–295-nm region, particularly at 288 and 293 nm (Figure 3B).

Preliminary Kinetic Observations. In both α-chymotrypsin and its zymogen, the changes in absorption around 300 nm (*i.e.*, in the main band of the pH-dependent spectra) are linked to a fast process (Bellon and Delaage, 1970). δ-Chymotrypsin and chymotrypsinogen were submitted to a fast pH jump, from pH 2 to pH 4.5, in a rapid mixing apparatus and the changes of either absorption or fluorescence were followed for both proteins.

In the case of chymotrypsinogen, the use of absorption at different wavelengths or of fluorescence does not allow the detection of any process occurring in the time range accessible to the stopped-flow technique, but solely a "burst;" this is in agreement with the results of Bellon and Delaage (1970) in our conditions of temperature and ionic strength. Owing to the difficulty of evaluating "instantaneous" changes which take place within the stopped-flow dead time, no further attempt to characterize this process was undertaken.

In the case of δ-chymotrypsin, two different kinetic processes could be seen upon a pH jump from pH 2 to pH 4.5: (a) a very fast reaction, also seen as a "burst," takes place at any of the wavelengths used for absorption experiments, 288, 293, and 300 nm; this fast process, which is apparently not detected in fluorescence experiments, has not been studied further; (b) a slower process, following first-order kinetics, is seen by either fluorescence (Figure 4) or changes in absorption at 288 and 293 nm (Figure 5); this slow reaction is not detected by absorption changes at 300 nm.

These results show that (at least) two different processes occur in δ-chymotrypsin upon a pH jump, and that the fluorescence changes observed at equilibrium seem to be linked to the slower of these processes.

Investigation of the Slow Process Occurring in δ-Chymotrypsin. Fluorescence changes were used for a further investigation of the slow process observed in δ-chymotrypsin, according to the pH jump method previously described (Garel and Labouesse, 1971). After a rapid pH change, the fluorescence change follows first-order kinetics (Figure 4), the rate constant of which depends on the final pH, and not on the initial one (actually pH 2 or pH 4.7 in this work). Figure 6 shows the dependence on the final pH of the rate constant of the fluorescence change; this rate constant seems to increase greatly when the final pH is lower than 3. The amplitude of the fluorescence change varies with the final pH according to the ionization curve of a group of $pK \sim 3$ (Figure 7), and accounts for the total fluorescence change observed at equilibrium. This indicates that no fast reaction contributes significantly to the change in fluorescence (Tanford, 1968), in agreement with the preliminary observations.

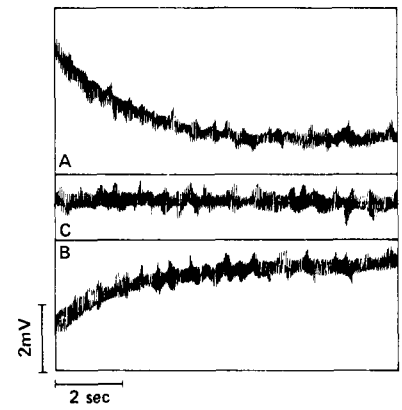

FIGURE 5: Oscilloscope tracings of the changes in absorption at 288 nm (curve A), 293 nm (curve B), and 300 nm (curve C) of δ-chymotrypsin upon a pH jump from pH 2 to pH 4.5. Final conditions as in Figure 4, except δ-chymotrypsin was 0.1 mg/ml. Total signal for the protein at pH 4.5 was 800 mV at all three wavelengths. Optical pathway, 2 cm. A change in signal of 2 mV corresponds to about 2.5×10^{-3} absorbance change.

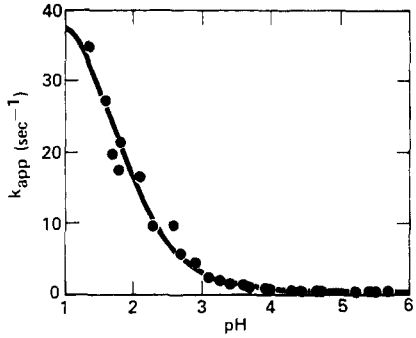

FIGURE 6: pH dependence of k_{app}, the rate constant of the changes in the fluorescence of δ-chymotrypsin upon pH jumps. Abscissa, final pH. The initial pH values were either pH 2.0 or pH 4.7.

As previously discussed (Garel, 1971; Garel and Labouesse, 1971), such a conformational change linked to the ionization of a single group can be represented by

$$\begin{array}{ccc} E & \overset{K}{\rightleftharpoons} & EH \\ k_1 \uparrow\downarrow k_{-1} & & k_2 \uparrow\downarrow k_{-2} \\ E' & \underset{K'}{\rightleftharpoons} & E'H \end{array} \qquad \text{(neutral pH)} \qquad \text{(acidic pH)}$$

where E and E′ are the enzyme conformations with respectively high and low fluorescence; H is the proton of the group having the pK ~ 3 at equilibrium; K and K' are the dissociation constants of this group in the E and E′ conformations (because of the experimental conditions, these protolytic reactions are taken as instantaneous equilibria (Hammes, 1968)); k_1, k_{-1}, k_2, and k_{-2} are the individual isomerization rate constants. The apparent rate constant of the fluorescence change at a final proton concentration H is given by $k_{app} = [(k_2H + k_1K)/(H + K)] + [(k_{-2}H + k_{-1}K')/(H + K')]$ (Garel and Labouesse, 1971). The data given in Figure 6 were analyzed according to this equation, and lead to the values listed in Table I.

The group pK ~ 3 at equilibrium has very different pK values in the E (pK ~ 1.5) and E′ (pK' ~ 4) conformations; protonation of this group favors the E′ conformation ($k_2/k_{-2} \sim 40$) and deprotonation of this group favors the E conformation ($k_{-1}/k_1 \sim 10$). The ionization of this critical group is therefore accompanied by an almost complete transition from the E′ to the E conformation.

TABLE I: Equilibrium and Rate Constants Pertaining to the Slow Transition of δ-Chymotrypsin at 15° (from Figure 6).

Phenomenon	Process	Constant
Apparent equilibrium pK	$E'H \rightleftharpoons E + H$	$pK_{app} = 3 \pm 0.2$
Proton dissociation	$EH \rightleftharpoons E + H$	$pK = 1.5 \pm 0.3$
	$E'H \rightleftharpoons E' + H$	$pK' = 4 \pm 0.3$
Isomerization reaction	$E \rightarrow E'$	$k_1 = (0.03 \pm 0.02)\ sec^{-1}$
	$E' \rightarrow E$	$k_{-1} = (0.25 \pm 0.10)\ sec^{-1}$
	$EH \rightarrow E'H$	$k_2 = (50 \pm 10)\ sec^{-1}$
	$E'H \rightarrow EH$	$k_{-2} = (1.2 \pm 0.4)\ sec^{-1}$
Conformation equilibrium	$E \rightleftharpoons E'$	$k_1/k_{-1} \sim 0.10$
	$EH \rightleftharpoons E'H$	$k_2/k_{-2} \sim 40$

Discussion

The Optical Changes Observed in δ-Chymotrypsin at Low pH Arise from More than One Process. Since δ-chymotrypsin is known not to aggregate or dimerize in the conditions of this work (Neet and Brydon, 1970; Miller *et al.*, 1971), these two phenomena do not interfere with our interpretation of the results.

The conformational change observed in δ-chymotrypsin at low pH appears to be complex; fluorescence and optical rotation changes do not follow identical pH dependences (Figures 1 and 2), and two kinetic processes have been detected upon a pH jump, which suggests that several pH dependent phenomena take place in the protein (Lumry *et al.*, 1966; Tanford, 1968). Among these, the ionization of a group of pK ~ 3 has been evidenced by fluorescence measurements at equilibrium (Figure 1) and by kinetics (Figure 7). Since fluorescence changes are involved only in the slower reaction, this reaction is likely to be controlled by the ionization of the group of pK ~ 3. This slow reaction is also accompanied by changes in absorption at 288 and 293 nm (Figure 5), but these changes do not account for the total variation observed at equilibrium, in contrast to the results of fluorescence studies. The pH dependent spectral changes seen at equilibrium in δ-chymotrypsin (Figure 3A) therefore contain contributions from the slow process (which depends on the pK of 3) and from other processes which take place in a much faster time scale. In the case of chymotrypsinogen the protein does not show such a slow reaction upon a pH jump and its fluorescence remains constant with pH. This indicates that the structural role of the group which has a pK around 3 in the enzyme is acquired during the activation process of the zymogen. A pK of 3 is likely to pertain to a carboxyl group (the normal pK of carboxyl groups in proteins is around 4 (Edsall and Wyman, 1958)), and we may already suspect this group of pK ~ 3 of being that of one of the two abnormal carboxyl groups of the enzyme, *viz.*, Asp-102 or Asp-194. But whatever the nature of this group is, in order to evaluate the change in δ-chymotrypsin properties which are specifically linked to its ionization, we need an estimate of the contribution of all other processes to the changes actually observed. We already know that fluorescence changes do not contain such extra contributions and that absorption changes do; we do not know anything about optical rotation changes.

Chymotrypsinogen may be Chosen as a Reference Protein to Estimate the Changes not Linked to the Ionization of the Group of pK ~ *3.* The major difference between the behaviors of δ-chymotrypsin and of its zymogen at low pH is the existence in the former and not in the latter of the slow reaction upon a pH jump. This difference seems to arise from the structural importance acquired by an ionizable group upon converting the zymogen into active enzyme. Support for this view that one ionizable group has different properties in the two proteins comes from the work of Fersht and Sperling (1973). These authors have studied the proton uptake upon denaturation by sodium dodecyl sulfate of both chymotrypsinogen and chymotrypsin in the pH range above pH 3, in order to monitor groups of abnormally low pK. They consistently observed that this proton uptake is lower by one proton for chymotrypsinogen than for chymotrypsin. This indicates that a group which has a normal (or close to normal) pK in the zymogen remains ionized at pH values below 3 in the enzyme. It has already been known that the activation process does not involve pK shifts in a large number of carboxyl groups (Marini and Martin, 1971), but these last findings show that a single ionizable group is significantly perturbed as far as pK changes are concerned. We shall therefore assume that the differences between the behaviors of δ-chymotrypsin and its zymogen at low pH all arise from the

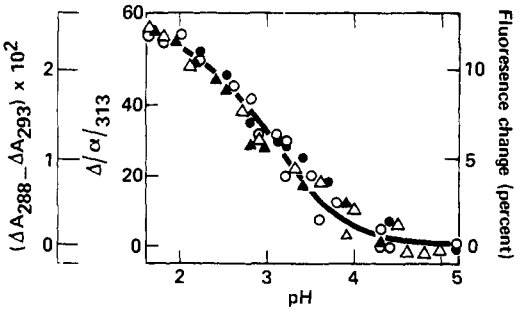

FIGURE 7: pH dependence of some parameters pertaining to δ-chymotrypsin after correction (see text): (△) specific optical rotation at 313 nm; (○) fluorescence observed at equilibrium; (●) amplitude of the slow fluorescence change, as determined from extrapolation to zero time of linear plots like that given in bottom curve of Figure 4; (▲) spectral changes, as determined by $(\Delta A_{288} - \Delta A_{293})$ from spectra like that given in Figure 3B. The solid line corresponds to the ionization curve of a group of pK = 3.

difference in the ionization properties of a single group, or in other words that ionizations of all groups, except for a single one, occur with identical pK's and optical changes in both proteins, in the considered pH range. The validity of this assumption and its relevance to known features of the structures of both proteins will be discussed below, but we can already support it with two different arguments: (a) at both 288 and 293 nm, the amplitudes of the absorption changes due to the slow process (Figure 5) are in agreement (in both sign and size) with the absorbancy difference between the changes seen in the enzyme and its zymogen (Figure 3B); at 300 nm where both proteins show similar absorption changes at equilibrium, no slow process is detected; (b) according to our assumption, the changes observed in δ-chymotrypsin can be corrected from those observed in the zymogen; the corrected fluorescence, absorption, and optical rotation changes all follow the same pH dependence, corresponding to the ionization of a group of pK ~ 3 (Figure 7). This result, which is *a priori* expected for the changes linked to the ionization of a single group (Tanford, 1961), is obtained despite the very different sizes of the corrections: no correction for fluorescence (Figure 1), a small one for optical rotation (Figure 2), and a large one for absorption (Figure 3). Assigning the changes observed in δ-chymotrypsin to the sum of what is observed in the zymogen and of changes linked to the group of pK ~ 3 thus appears to be consistent with the experimental results. These changes are not a direct effect of the ionization of the group of pK ~ 3, but of the conformational change linked to it.

The Changes Linked to the Ionization of the Group of pK ~ *3 can be Compared to Those Linked to the Ionization of the α-Amino Group of Ile-16* (pK ~ *9*). Some of the features of the conformational change that we have assigned to the ionization of the group of pK ~ 3 in δ-chymotrypsin have been listed in Table II, together with the changes linked to the ionization of the α-amino group.[2] Both groups appear to control very similar changes of chymotrypsin conformation.

The relationship between protein conformation and ligand (substrate or inhibitor) binding seems also to be the same at either acidic or alkaline pH. Both the acidic (Delaage *et al.*, 1968) and alkaline (Fersht and Requena, 1971) conformational changes are accompanied by a loss of affinity for proflavine, and in both cases the presence of an excess of ligand seems to

[2] In δ-chymotrypsin Ala-149 is engaged in an amide bond; the α-amino group of this residue is therefore not involved in any of the change discussed here. In α-chymotrypsin, the α-amino group of Ala-149 has been implicated in the changes in properties observed at high pH (Valenzuela and Bender, 1971).

TABLE II: Comparison between the Magnitudes of the Changes Observed in δ-Chymotrypsin upon Ionization of the Group of pK ~ 3 at Acidic pH, and Those Observed in Acetylated δ-Chymotrypsin upon Ionization of the α-Amino Group of Ile-16 at High pH.

Parameter observed	Acidic pH Transition (pH 2–4)	Alkaline pH Transition (pH 8–10)
Specific optical rotation change at 313 nm	(58 ± 4)° [a]	(54 ± 3)° [b]
Relative fluorescence change (%)	11 ± 1 [a]	10 ± 1 [c]
Position of the main bands of spectral changes	280 nm [d] 288 nm 293 nm	279 nm [c] 288 nm 292 nm
Absorption change between 288 and 293 nm (Δε)	(7 ± 3) 10^2 [d]	(10 ± 3) 10^2 [c]
Apparent rate of isomerization at final pH 5.5	0.25 sec^{-1}	0.23 sec^{-1}

[a] From Figure 7. [b] From Karibian *et al.* (1969). [c] From Garel and Labouesse (1971). [d] From Figure 3B.

reverse the conformational change undergone by the free enzyme (Parker and Lumry, 1963; Hess *et al.*, 1970; Garel and Labouesse, 1970). Acylation of Ser-195 by diisopropyl fluorophosphate prevents the enzyme transition at both acidic (Parker and Lumry, 1963; Biltonen *et al.*, 1965b) and alkaline (Oppenheimer *et al.*, 1966) pH's, and, accordingly, we could not detect any slow reaction upon a pH jump in diisopropylphosphoryl-δ-chymotrypsin (S. Epely, unpublished results).

Chymotrypsin activity has been followed down to pH's of the order of 2 (*i.e.*, through the pH range of the conformational change described here), and its pH dependence can be accounted for on the basis of a single ionizable group with a pK around 7 (Kezdy *et al.*, 1964). We have explained previously why steady-state measurements of activity are not sensitive to the presence of such a conformational change, except during the very early part of the reaction (Garel and Labouesse, 1973): the binding of the substrate displaces this equilibrium in favor of the conformation having the higher affinity. Presteady-state measurements of activity will be sensitive to this conformational change, provided that the rate constant of the reaction used to monitor activity is at least of the same order of magnitude as that of the substrate-promoted isomerization mentioned above (Fersht, 1972; Garel and Labouesse, 1973). The highest rate constant of a chymotrypsin-catalyzed reaction, that of acylation by a *p*-nitrophenyl ester of an aromatic amino acid, is about 5 × 10^4 sec^{-1} (Zerner and Bender, 1963; Philipp and Bender, 1973; Renard and Fersht, 1973). Assuming that this rate is governed by a pK of 7, its value would be 5 sec^{-1} at pH 3; the rate constant of isomerization of δ-chymotrypsin at this pH is 3 sec^{-1} at 15° (Figure 6) and 12 sec^{-1} at 25° (S. Epely, unpublished results). The comparison of those values suggests that the conformational change reported here may perhaps be evidenced by activity measurements, but only by using good substrates. But even if the catalytic activity of chymotrypsin at low pH is expected to be only hardly sensitive to the conformational change, the affinity is expected to decrease significantly below pH 4, according to the same pattern as that observed above pH 8 (Brandt *et al.*, 1967).

The Group of pK 3 may be Identified with the β-Carboxyl

Group of Asp-194. X-Ray crystallography has evidenced two buried carboxyl groups in chymotrypsin: the β-carboxyl groups of Asp-194 (which interacts with the α-amino of Ile-16 (Matthews *et al.*, 1967)) and of Asp-102 (which interacts with His-57 so as to form the "charge relay system") (Blow *et al.*, 1969). Chemical modification studies have shown that blocking most of the carboxyl groups does not destroy completely the activity of chymotrypsin (or the potential activity of its zymogen) (Carraway *et al.*, 1969; Abita and Lazdunski, 1969; Ghelis, 1971; Fersht and Sperling, 1973) whereas further blocking of two carboxyl groups in denaturing conditions inactivates the enzyme (Carraway *et al.*, 1969; Abita and Lazdunski, 1969; Ghelis, 1971; Fersht and Sperling, 1973); one of these critical groups was identified as Asp-194 (Carraway *et al.*, 1969; Abita and Ladzunski, 1969), the other was assumed to be Asp-102. Fersht and Sperling (1973) have found a difference of one abnormal carboxyl group between modified chymotrypsin and chymotrypsinogen where only Asp-102, Asp-194, and two to three other carboxyl groups were free to ionize.

The involvement of Asp-102 as the group of p$K \sim 3$ controlling the conformational change in δ-chymotrypsin has become very unlikely since the findings of Robillard and Schulman (1972): these authors observe that the nuclear magnetic resonance signal of the proton bridging Asp-102 and His-57 is the same in δ-chymotrypsin and in its zymogen, and furthermore is not affected by lowering the pH down to 3.

All the results cited above point toward Asp-194 as the best candidate for a carboxyl group endowed with a crucial role in chymotrypsin since Asp-102 can be ruled out. Moreover we have found that the group of p$K \sim 3$ and the α-amino group of Ile-16 control very similar conformational changes (Table II), which we believe to arise from the same structural feature, namely the Ile-16–Asp-194 salt bridge. We are aware that the proof that the group of p$K \sim 3$ is the β-carboxyl group of Asp-194 requires a direct and unequivocal identification based on chemical methods; nevertheless the set of evidence presented so far in favor of the assignment of p$K \sim 3$ to Asp-194 seems to us sufficient enough to make this assignment highly probable.

Relevance of the Present Interpretation to Known Features of Chymotrypsin and Chymotrypsinogen Structures and to the Activation Process. We have assumed that the only difference between the behavior of δ-chymotrypsin and its zymogen at low pH is a change in the properties of a single group, that of the Asp-194 side chain. A comparison of the crystal structures of the two proteins (Kraut, 1971; Blow, 1971; Wright, 1973) shows that only one carboxyl group seems to be significantly perturbed by the activation process, that of Asp-194, which moves by 4 Å. In the zymogen Asp-194 interacts with His-40 (Freer *et al.*, 1970; Kraut, 1971), and the same interaction may also exist in chymotrypsin when Asp-194 is not in a salt linkage with Ile-16, *i.e.*, in the E′ conformation. The pK of Asp-194 in the E′ conformation has been found to be around 4[3] (Table I), close to that of a carboxyl group in an aqueous medium (Edsall and Wyman, 1958); this indicates that the His-40–Asp-194 interaction must be very weak (at least as compared to the Ile-16–Asp-194 interaction which brings the pK of Asp-194 down to 1.5 in the E conformation (Table I)). Fersht (1972) also finds that the His-40–Asp-194 interaction in chymotrypsin (if any) would be about six times weaker than the Ile-16–Asp-194 salt bridge; the omission of taking any His-40–Asp-194 interaction into account in our procedure is therefore unlikely to introduce large errors.

It has been reported that the ionization state of (some of) the surface carboxyl groups has an influence on the enzyme conformation, as seen from the ability to bind proflavine (Fersht, 1972). However, the affinity of chymotrypsin for proflavine is known to decrease a low pH (Fersht and Requena, 1971; Brandt *et al.*, 1967), and it is possible that these experiments were not done in saturating conditions.[4]

A last point seems to us worth mentioning: the first-order rate constant for the activation of chymotryspinogen, 0.18 sec^{-1} at 1° (Abita *et al.*, 1969), appears to be close to the rate constant of formation of the E conformation (the one in which the Ile-16–Asp-194 interaction can build up), 0.25 sec^{-1} at 15°; this similarity suggests that the rate-limiting step of the activation process may be the structural rearrangement which yields the E conformation of the enzyme upon the formation of the Ile-16–Asp-194 ion pair. It is known that the rates of activation of either chymotrypsinogen B (Abita *et al.*, 1969) or chymotrypsinogen A modified on carboxyl groups (Carraway *et al.*, 1969; Lazdunski *et al.*, 1970) (both differing from unmodified chymotrypsinogen A by their charge distributions (Smillie *et al.*, 1968)) are about ten times faster than that of chymotrypsinogen A. The study of the rates of isomerization, upon ionization of Asp-194, of either chymotrypsin B or chymotrypsin whose surface carboxyl groups have been modified should provide an experimental test for our hypothesis, *viz.*, that the refolding of chymotrypsinogen upon cleavage of the Arg-15–Ile-16 peptide bond is the same as the one observed in chymotrypsin when forming the Ile-16–Asp-194 interaction.

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References

Abita, J. P., Delaage, M., Lazdunski, M., and Savrda, J. (1969), *Eur. J. Biochem. 8*, 314–324.

Abita, J. P., and Lazdunski, M. (1969), *Biochem. Biophys. Res. Commun. 35*, 707–712.

Bellon, B. R., and Delaage, M. (1970), *Eur. J. Biochem. 17*, 43–48.

Biltonen, R., and Lumry, R. (1969), *J. Amer. Chem. Soc. 91*, 4256–4264.

Biltonen, R., Lumry, R., Madison, V., and Parker, H. (1965a), *Proc. Nat. Acad. Sci. U. S. 54*, 1018–1025.

Biltonen, R., Lumry, R., Madison, V., and Parker, H. (1965b), *Proc. Nat. Acad. Sci., U. S. 54*, 1412–1419.

Biltonen, R., Schwartz, A. T., and Wadsö, I. (1971), *Biochemistry 10*, 3417–3423.

[3] The pK of Asp-194 in the E′ conformation appears to be close to normal although this residue needs denaturing conditions to be chemically modified in both chymotrypsinogen (Ladzunski *et al.*, 1970; Carraway *et al.*, 1969) and the E′ conformation (Ghelis, 1971), and therefore looks buried. This can be due to some steric factors of the active site which would allow Asp-194 to be in contact with a water molecule and not with a bulky carbodiimide reagent usually used for carboxyl groups.

[4] Proflavine is a positively charged molecule at neutral and acidic pH (Glazer, 1965); it is possible that its binding on chymotrypsin is affected by changes of the global charge carried by the protein. This binding would decrease at low pH owing to the increasing positive charge of the protein upon protonation of the surface carboxyl groups. Such a decrease in binding would then be nonspecific and not controlled by any conformational effect.

Blow, D. M., (1971), *Enzymes, 3rd Ed. 3,* 185–212.
Blow, D. M., Birktoft, J. J., and Hartley, B. S. (1969), *Nature (London) 221,* 337–340.
Brandt, K. G., Himoe, A., and Hess, G. P. (1967), *J. Biol. Chem. 242,* 3973–3982.
Bridges, J. W., and Williams, B. T. (1968), *Biochem. J. 107,* 225–237.
Carraway, K. L., Spoerl, P., and Koshland, D. E. (1969), *J. Mol. Biol. 42,* 133–137.
Delaage, M., Abita, J. P., and Lazdunski, M. (1968), *Eur. J. Biochem. 5,* 285–293.
Dixon, G. H., and Neurath, H. (1957), *J. Biol. Chem. 225,* 1049–1059.
Edsall, J. T., and Wyman, J. (1958), in Biophysical Chemistry, Vol. 1, New York, N. Y., Academic Press, p 406.
Fersht, A. R. (1972), *J. Mol. Biol. 64,* 497–509.
Fersht, A. R. (1973), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 29,* 283–285.
Fersht, A. R., and Requena, Y. (1971), *J. Mol. Biol. 60,* 279–290.
Fersht, A. R., and Sperling, J. (1973), *J. Mol. Biol. 74,* 137–149.
Freer, S. T., Kraut, J., Robertus, J. D., and Xuong, Ng. H. (1970), *Biochemistry 9,* 1997–2009.
Garel, J. R. (1971), Thesis, Université de Paris, Orsay, France.
Garel, J. R., and Labouesse, B. (1970), *J. Mol. Biol. 47,* 41–56.
Garel, J. R., and Labouesse, B. (1971), *Biochimie 53,* 9–16.
Garel, J. R., and Labouesse, B. (1973), *Eur. J. Biochem. 39,* 293–300.
Ghelis, C. (1971), Thesis, Université de Paris, Orsay, France.
Ghelis, C., Garel, J. R., and Labouesse, J. (1970), *Biochemistry 9,* 3902–3913.
Ghelis, C., Labouesse, J., and Labouesse, B. (1967), *Biochem. Biophys. Res. Commun. 29,* 101–106.
Glazer, A. N. (1965), *Proc. Nat. Acad. Sci. U. S. 54,* 171–176.
Gros, C., and Labouesse, B. (1969), *Eur. J. Biochem. 7,* 463–470.
Hammes, G. G. (1968), *Advan. Protein Chem. 23,* 1–57.
Hess, G. P. (1971), *Enzymes, 3rd Ed. 3,* 213–248.
Hess, G. P., Mc Conn, J., Ku, E., and Mc Conkey, G. (1970), *Phil. Trans. Roy. Soc. London, Ser. B 257,* 89–104.
Karibian, D., Laurent, C., Labouesse, J., and Labouesse, B. (1969), *Eur. J. Biochem. 5,* 260–269.
Kezdy, F. J., Clement, G. E., and Bender, M. L. (1964), *J. Amer. Chem. Soc. 86,* 3690–3696.
Kraut, J. (1971), *Enzymes, 3rd Ed. 3,* 165–183.
Lazdunski, M., Delaage, M., Abita, J. P., and Vincent, J. P. (1970), in Structure-Function Relationships of Proteolytic Enzymes, Desnuelle, P., Neurath, H., and Ottesen, M., Ed., Copenhagen, Munksgaard, pp 42–55.
Lumry, R., Biltonen, R., and Brandts, J. F. (1966), *Biopolymers 4,* 917–944.
Marini, M. A., and Martin, C. J. (1971), *Eur. J. Biochem. 19,* 162–168.
Matthews, B. W., Sigler, P. B., Henderson, R., and Blow, D. M. (1967), *Nature (London) 214,* 652–656.
Mc Conn, J., Fasman, G. D., and Hess, G. P. (1969), *J. Mol. Biol. 39,* 551–562.
Miller, D. D., Harbett, T. A., and Teller, D. C. (1971), *Biochemistry 10,* 4641–4648.
Neet, K. E., and Brydon, S. E. (1970), *Arch. Biochem. Biophys. 136,* 223–227.
Oppenheimer, H. L., Labouesse, B., and Hess, G. P. (1966), *J. Biol. Chem. 242,* 2720–2730.
Parker, H., and Lumry, R. (1963), *J. Amer. Chem. Soc. 85,* 483–484.
Philipp, M., and Bender, M. L. (1973), *Nature (London), New Biol. 241,* 44.
Renard, M., and Fersht, A. R. (1973), *Biochemistry 12,* 4713–4718.
Robillard, G., and Schulman, R. G. (1972), *J. Mol. Biol. 71,* 507–511.
Robinson, N. C., Neurath, H., and Walsh, K. A. (1973), *Biochemistry 12,* 420–426.
Rupley, J., Dreyer, W., and Neurath, H. (1955), *Biochim. Biophys. Acta 18,* 162–163.
Sigler, P. B., Blow, D. M., Matthews, B. W., and Henderson, R. (1968), *J. Mol. Biol. 35,* 143–164.
Smillie, L. B., Furka, A., Nagabhushan, N., Stevenson, K. J., and Parkes, C. O. (1968), *Nature (London) 218,* 343–346.
Tanford, C. (1961), *J. Amer. Chem. Soc. 83,* 1628–1634.
Tanford, C. (1968), *Advan. Protein Chem. 23,* 121–282.
Valenzuela, P., and Bender, M. L. (1971), *J. Amer. Chem. Soc. 93,* 3783–3784.
Wright, H. T. (1973), *J. Mol. Biol. 79,* 1–11.
Zerner, B., and Bender, M. L. (1963), *J. Amer. Chem. Soc. 85,* 356–358.