IDENTIFICATION OF THE IONISING GROUP CONTROLLING THE ACTIVE CONFORMATION OF α-CHYMOTRYPSIN IN ALKALINE pH [*]

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Received August 29, 1967

An ionising group, with an apparent pK of 8.5-9 has been implicated in the control of conformation (Moon et al., 1965, Oppenheimer et al., 1966) and in the control of activity (Bender et al., 1963, Oppenheimer et al., 1966) of chymotrypsin (CT). This group seems, very likely, to be involved in the rate limiting step, above pH 8, in amide hydrolysis (Bender and Clement, 1963, Himoe and Hess, 1966).

Several hypotheses have been made as for the nature of this group but the most recent data point to the α-amino group of N-terminal isoleucine (Oppenheimer et al, 1966), which appears when chymotrypsinogen is converted into chymotrypsin (Desnuelle, 1960). It is known that none of the ε-amino groups of lysines nor the α-amino group of the N-terminal 1/2 cystine nor the hydroxyl group of the two exposed tyrosines appear to be essential for activity since it has been possible to prepare an active acetyl-chymotrypsin in which all these groups are acetylated (Labouesse et al., 1964). However reacetylation of such an acetylated chymotrypsin results in loss of activity.

It was of interest to know a) if this loss of activity corresponds to the acetylation of one or several groups of the enzyme molecule; b) which is the residue (or residues) in the primary structure acylated in the course of this reacetylation.

[*] This investigation has been supported by grants from the Centre National de la Recherche Scientifique (RCP n°23 and E. R. A. n°34).

Evidence presented in this paper shows that acetylation of α-CT (in which all of the amino groups except one : the N-terminal α-amino group of isoleucine, and all of the phenolic hydroxyls of exposed tyrosines are already acetylated with cold reagent) with radioactive acetic anhydride, leads to incorporation of one equivalent of ^{14}C labelled acetate per mole of protein. The degradation of the labelled protein under mild conditions, demonstrates that the radioactive label is carried by the N-terminal isoleucine.

Labelled ^{14}C-acetic anhydride, (Ac_2O), has been used for the reacetylations. The starting material was purified acetyl-α-CT prepared by the method described previously (Oppenheimer et al., 1966) from chymotrypsinogen (Worthington, lot CG 763) acetylated with cold Ac_2O. The determination of the nature and the quantity of free amino groups in the starting enzyme was performed using condensation of the protein with FDNB in very similar conditions as those described by Labouesse and Gervais (1967). The results of these determinations are given in Table I. Reacetylation of acetyl-α-CT with ^{14}C-Ac_2O was conducted in the same conditions as the first acetylation. The activity of the enzyme towards acetyl-tyrosine ethyl ester (ATEE) was checked before and after the second acetylation (Table I). At the end of this reacetylation when excess of reagent was hydrolysed, the protein was treated at 25° with 0.5 M hydroxylamine at pH 7.5 over a period of 30 minutes , then dialysed. The activity and radioactivity were then measured again (Table I). The hydroxylamine treatment was intended to check if the incorporation of ^{14}C and the loss of enzymatic activity towards ATEE was not due only to the formation of a labile covalent bond between the ^{14}C-acetate and some residue of the protein, for instance the "active" serine or the "active" histidine. Results of Table I show that there is incorporation of 1.0 equivalent of ^{14}C per mole of protein in acetyl-α-CT upon reacetylation and that the group which has been acetylated is not sensitive to a 0.5 M hydroxylamine treatment at pH 7.5, which certainly deacylates active serine, histidine and exposed tyrosines . It was checked that a longer hydroxylamine treatment in the same conditions does not alter the incorporated radioactivity and the enzymatic activity of the protein.

TABLE I

	NH_2terminal[a]	$k_{obs.}$ ATEE(sec^{-1})[b]	^{14}C DPM per μM[c]
Acetyl-CT-gen	none		
Acetyl- -CT	Ile	210	
Acetyl-(acetyl-^{14}C-$_{Ile}$)-CT	none	18	1.4×10^5
Acetyl-(acetyl-^{14}C-Ile)-CT, after treatment 0.5 M NH_2OH	none	18	1.4×10^5
P_1-^{14}C			1.4×10^5
P_2-^{14}C			1.4×10^5

a) Determined as DNP-aminoacids
b) ATEE, 1.6×10^{-2}M, pH 8.0, KCl 0.1M, 25°.
c) Measured in a Packard Tri-Carb Liquid Scintillator.
The specific radioactivity of acetic anhydride was 1.4×10^5 DPM per μmole.

The radioactive protein was degraded, in order to demonstrate the residue to which the labelled acetate has been attached:

I) The protein (total R.: 2.8×10^5 DPM, 2 μmoles, Spec. R.: 1.4×10^5 DPM per μmole) was reduced by β-mercaptoethanol and the liberated -SH groups were blocked with ethylenimine according to Raftery and Cole (1966) to increase the number of susceptible bonds of the polypeptide chain to trypsin digestion (all lysine residues are blocked by acetylation, leaving only three susceptible -Arg-X- bonds). After dialysis [x], the protein was purified by fractionation on Sephadex G 25 column. The radioactive fraction (total R.: 2.75×10^5 DPM) was submitted to high voltage paper electrophoresis (2,000 V, pyridine-acetate buffer pH 6.5 and 5.5, for 70 minutes). The single radioactive band (revealed by autoradiography) was eluted and digested with trypsin, (trypsin treated with TPCK according to Kostka and Carpenter, 1964);

II) The tryptic digest (Table II) was fractionated on a Sephadex G 25 column. The radioactive peak (Total R.: 2.4×10^5 DPM) was purified by

[x] Dialysis eliminates the oligopeptide corresponding to the sequence Cys_1-Leu_{13}- of the A-chain of CT, but, as the initial radioactivity is recovered after the Sephadex column, this peptide should not carry any ^{14}C.

high voltage paper electrophoresis (under conditions already described) and by thin layer chromatography; radioautography showed one radioactive band. After purification, the radioactive product P_I-^{14}C was not reactive to ninhydrine and had no absorption at 280 mμ. The composition of the isolated peptide P_I-^{14}C after total acid hydrolysis and aminoacid analysis (Technicon Aminoacid Analyser) is shown in Table II.

This composition corresponds to the N-terminal sequence of the B-chain of CT (Hartley, 1964, Matthews et al., 1967):

Ile_{16}-Val-Asn-Gly-Glu-Glu-Ala-Val-Pro-Gly-Ser_{26}-

of the sequence of the chymotrypsinogen. Threonine and arginine are absent in the radioactive isolated material. This indicates that the starting material was devoid of α-CT with free N-terminal C-chain, which also would have been acetylated during the radioactive reacetylation. The finding of this peptide as a result of tryptic digestion is unexpected: the -Ser_{26}-Try_{27}- bond should not be susceptible to trypsin.

The purified peptide P_1-^{14}C was submitted to chymotryptic digestion.

TABLE II

Composition of isolated radioactive peptides P_1-^{14}C and P_2-^{14}C.

Aminoacids	P_1 [a] Molar ratio		P_2 [d] Molar ratio	
	Theoretical [b]	Observed [c]	Theoretical [b]	Observed [c]
Aspartic	1	1.2	1	0.9
Serine	1	1.3		
Glutamic	2	2.6		
Proline	1	1.0		
Glycine	2	2.0		
Alanine	1	1.0		
Valine	2	2.7	1	0.7
Isoleucine	1	1.0	1	1.0

a) P_1-C^{14}: peptide isolated after tryptic digestion (enzyme-protein ratio 1/100, in borate buffer-$CaCl_2$ 0.01 M, pH 7.5 for 32 h. at 37°, with stirring).

b) Calculated for the N-terminal sequence of acetylated- α-CT.

c) Calculated taking the molar ratio of acetylated -isoleucine as 1.0.

d) P_2-^{14}C : peptide isolated after chymotryptic digestion of P_1-^{14}C (enzyme protein ratio 1/100, pH 7.5 for 15 h. at 37° in borate buffer).

III) The chymotryptic hydrolysate (Table II) was purified by thin layer electrophoresis (200 V, acetate-pyridine buffer pH 6.5 and 5.5, for 40 minutes) and thin layer chromatography. A product, P_2-^{14}C, unreactive to ninhydrin (Total R.: 2.38x10^5 DPM) was recovered and submitted to total acid hydrolysis. The composition of P_2-^{14}C according to the data obtained by aminoacid analysis is shown in Table II and corresponds to the unique N-terminal sequence of the B-chain of CT, Ile_{16}-Val_{17}-Asn_{18}- in the sequence of chymotrypsinogen. The quantity of isoleucine in P_2-^{14}C was 1.7 μmole and the radioactivity 2.38x10^5 DPM. This leads to a specific R.: 1.4 x10^5 DPM per μmole of isoleucine.

These experiments demonstrate that:

a) reacetylation of acetyl-α-CT, which leads to a decrease in activity of CT towards ATEE brings about specifically the acetylation of the α-amino-group of N-terminal isoleucine;

b) 85 % of the initial ^{14}C-acetate incorporated in the enzyme are recovered in the product P_2-^{14}C;

c) the specific radioactivity of the isolated ^{14}C-acetyl-peptide is comparable to the initial specific radioactivity of the ^{14}C-acetate incorporated in the enzyme.

These results clearly show that the loss of activity of acetyl-α-CT towards ATEE upon reacetylation is somehow linked to the acetylation of the α-aminogroup of the N-terminal isoleucine.

The kinetics of the incorporation of ^{14}C-acetate and of the loss of activity towards several specific substrates, is now under investigation. This decrease in activity may be due either:

1) to a change of affinity of the enzyme towards its substrates (changes in K_m)

2) to a change in the efficacity of the enzyme (change in k_{cat}),

3) to a loss of the active site.

To distinguish between the three proposed hypotheses, it is necessary to determine the kinetics of hydrolysis of several specific substrates (esters and amides) with the reacetylated acetyl-α-CT and titrate the active site of the modified enzyme.

Preliminary experiments with p-nitrophenyl acetate indicate that in acetyl-(^{14}C-acetyl-$_{Ile}$)-CT prepared under the conditions described in this paper more than 50 % of the active site is still present. If analogy

between CT and trypsin may be drawn, our observation does not seem in agreement with recent results of Scrimger and Hofmann (1967) who showed that in the case of trypsin, destruction of the N-terminal isoleucine is accompanied by loss of the active site of the enzyme. On the other hand, recent data of Himoe et al. (1967), Bender et al. (1966) and Johnson and Knowles (1967) suggesting that the affinity of CT towards substrates and inhibitors is decreased by increasing the pH above 8, would better explain our experimental results: the authors attribute this change in affinity to the deprotonation of the previously implicated α-NH_2 group of the N-terminal isoleucine. In the case of acetylation, the α-aminogroup of isoleucine is no longer ionisable, in the alkaline pH range; this can be the reason for the decrease in activity without impairing the active site. Furthermore the fact that the change with pH of the optical rotation, which follows the titration of the N-terminal isoleucine (Oppenheimer et al., 1966), is not observed for acetyl-(^{14}C-acetyl$_{Ile}$)-CT [*] supports the hypothesis in favor of a change in affinity of the enzyme for its substrates; this affinity is controlled by the enzyme conformation which is itself dependent on isoleucine ionisation.

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[*] The specific rotation of acetyl-(^{14}C-acetyl-$_{Ile}$)-CT is constant over the pH range 7-10 and is similar to the specific rotation of acetyl-chymotrypsinogen at pH 10 (J. Labouesse, unpublished data).