THE TERMINAL CARBOXYL GROUPS OF INSULIN

by

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According to SANGER[1] and TISELIUS AND SANGER[2] the insulin sub-molecule of molecular weight 12000 consists of four open peptide chains, connected by the dithio bridges of cystine. Two of these chains end in glycyl residues, the two other having phenylalanine as terminal groups.

As yet there are no indications which amino acid residues are present at the opposite end of these four chains, *i.e.*, those with a free carboxyl group or with a carboxy-amide group. The partial proteolysis under the influence of carboxypeptidase appeared to offer a useful method to tackle this problem. Its use for this purpose has frequently been discussed in literature. In the case of glutathione, the terminal group has actually been determined with the aid of this enzyme[3].

The difficulties in the evaluation of the results obtained with carboxypeptidase arise from the fact that a sufficient number of examples of its action on simple polypeptides is still lacking. Therefore the general conclusion that carboxypeptidase liberates only amino acid residues with a free carboxyl and a bound α-amino-group is not fully convincing, and some exceptions to this rule are already present amongst the few peptides investigated. BERGMANN AND HOFMANN[4] observed that carbobenzoxyglycylglutamic acid was only slowly attacked, whereas ε-hippuryllysine is split quite readily, although it has a free α-amino group.

Furthermore it is not at all a well established fact that carboxypeptidase is able to hydrolyse the terminal groups of intact proteins. Its action on casein, digested with trypsin, is well known and sometimes used for assay purposes, but so far no experiments with an intact native protein have been recorded.

We have now demonstrated that carboxypeptidase attacks insulin, splitting off one or more terminal groups. These have been identified, offering some further insight into the fine structure of this protein.

EXPERIMENTAL

The insulin used was crystalline material of our own manufacture, which in the air dry state showed an activity of 25 international units per mg. The moisture content was 9%.

The carboxypeptidase was prepared from cattle pancreas according to ANSON[5]. It was recrystallized three times and its activity was checked with chloroacetyl tyrosine. It was stored in the ice box as an aqueous suspension. The course of the hydrolysis was followed by the determination of the free amino groups with the manometric VAN SLYKE apparatus.

The determination of the amino groups liberated offers certain difficulties, because the value for the free amino groups in the intact insulin molecule is dependent on the

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experimental conditions. The reaction time is very important as SANGER[1] rightly pointed out, but the temperature at which the reaction takes place is of no less influence. Our zero value, representing the free amino groups of the intact molecule, varied from 6.0 to 8.0 aequivalent NH_2 per mol insulin of weight 12000. The highest value was obtained at 30°, the lowest at 16° C, both with a reaction time of 5 minutes. As the temperature of the VAN SLYKE reaction chamber cannot easily be controlled, it was decided to take the increase of the amino groups rather than the absolute value as a criterion. As during the course of one experiment the room temperature, which is also the temperature of the reaction chamber, did not vary greatly, amino acid determinations of one run were comparable.

For an experiment 500 mg of the insulin were dissolved in 10 ml of water, by adjusting the p_H to 7.7 with the aid of 0.1 N NaOH. No buffers were added in order to avoid interference in the partition chromatography of the digestion mixture. To this solution 0.2 ml of a suspension containing 4 mg of the carboxypeptidase crystals were added. During the reaction a temperature of 37° was maintained. As represented in Fig. 1, the VAN SLYKE amino nitrogen value of the solution increased steadily during the first 4 hours of the experiment and than slowed down considerably, 3.1 amino groups having been set free. In one experiment the reaction was stopped after 2.6 amino groups had been liberated, by the adjustment of the p_H to 5. The solution was filtered from the precipitate formed, and subsequently ultrafiltered through a cellophane membrane pretreated with a 100W/V % solution of zinc chloride to increase the porosity.

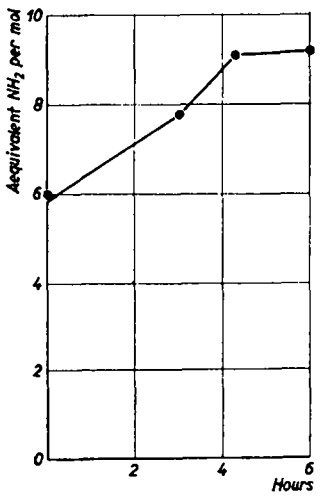

Fig. 1. 500 mg insulin digested with 4 mg carboxypeptidase. Volume 10.2 ml p_H 7.7. Temperature 37°. Abscissae: time in hours. Ordinate: number of NH_2 groups per submol of insulin (M.W. 12000).

In the ultra filtrate the total (Kjeldahl) nitrogen amounted to 3.80 mol per mol of insulin employed. 1.24 mol was due to ammonia, and the remaining 2.56 mol agreed well with the direct amino nitrogen determination in the ultra filtrate of 2.6 mol. Hence within the experimental error all amino groups liberated during the proteolysis are accounted for in the ultra filtrate, which, besides some ammonia, does not contain any other form of nitrogen. Therefore the enzyme actually liberated amino acids from the end of the peptide chain, and no peptides have been formed. The ammonia liberated is not dependent on the enzyme action.

For the analysis of the amino acids present in the ultra filtrate use has been made of partition chromatography on paper. Phenol and collidine have been employed as moving solvents. As suggested by WINSTEN[6], phenol shaken with saturated NaCl solution has been used in the later experiments, thus avoiding water logging of the paper.

The paper selected after many failures was type V 262 of G. SCHUT, Heelsum, Holland; it gives excellent straight sharp liquid fronts and almost no background colour after spraying with ninhydrin solution.

Only one amino acid, *alanine*, has been detected in the ultra filtrate of this insulin digest. Another digestion was continued for 6 hours, to the point where 3.16 amino groups were liberated according to the VAN SLYKE determination. Now, apart from

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alanine, some weak spots appeared. To determine these the solution was concentrated *in vacuo*. As the increased electrolyte content caused some water logging of the paper and an obvious shift of the now very strong alanine spot, the solution was desalted in the apparatus of CONSDEN, GORDON, AND MARTIN[7], and subsequently analysed with good results. Glycine, valine, the leucine group, and traces of tyrosine and of the mono amino dicarboxylic acid group were detected.

As a further breakdown of the molecule could not reveal anything of interest, the digestion was not continued any further.

A sample of the insulin has been investigated after 1.80 mol of alanine had been liberated. The solution was adjusted to p_H 3 to stop the enzyme action and biologically assayed in rabbits. 15% of the original activity was still left. At p_H 5 no crystal formation was observed, only a heavy amorphous precipitate appeared. A solution of p_H 2.8 in 0.01 N sodium sulphate showed only a slight precipitate after 20 minutes in a boiling water bath. The original insulin under these conditions shows a bulky precipitate after a few minutes' heating.

DISCUSSION

From these investigations it appears certain that of the amino acid residues with a free carboxyl group, present in an insulin submolecule, one consists of alanine. One or two of the others remaining might also be alanyl groups, but these experiments cannot bring a clear-cut decision in this respect. An alternative is a di-alanyl-alanine group at the end of one chain and groups which are much more slowly attacked by carboxypeptidase on the other ends. An intermediate configuration, with one alanyl-alanine group and one alanine group on two of the chains, is also possible.

When the alanine has been split off a considerable number of amino acids appear simultaneously in the solution at a greatly reduced rate. The technique did not permit to determine these quantitatively because of the very minute amounts present and it is questionable if it would be useful to explore this phase in more detail, further exact location of the involved amino acids in the chain remaining an impossible task.

The fact that the insulin did not show any tendency to crystallize after a short incubation is not very surprising: the precipitate is certainly not homogeneous, unattacked insulin and molecules with from one to three alanine groups missing being present simultaneously.

The lack of the property to form a heat precipitate after only a few amino acids have been removed from the chain will have to be taken in account in the explanation of the heat-precipitation of the intact molecule. However, as ANSON[8] rightly pointed out, it is still too early to draw a picture of the denaturation mechanism in detail, and the fact mentioned is difficult to reconcile with any of the existing theories on the subject.

In principle the application of this method to other proteins, in order to elucidate the nature of the terminal group, will be possible. However it should be borne in mind that with insulin circumstances were particularly favourable. In the first place the insulin sub-unit is rather small. Nevertheless it was necessary to detect one amino acid, which, assuming an average molecular weight of 100, could be expected to be present in a quantity of 0.8% of the weight of the protein employed. Secondly it was found that the first three amino acids liberated were identical, making the amount of the amino acid to be analysed at once three times as large as originally anticipated. Thirdly the check

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on the course of the enzyme reaction by analysis of total N and amino-N in the filtrate would have been irrelevant had the liberated amino acid contained other than amino nitrogen.

All three factors considerably facilitated the course of this investigation, but none is essential to the successful use of the method employed. A further analysis seems possible along the lines indicated by TISELIUS AND SANGER[2], *i.e.*, by oxidation of the disulphide bridges, isolation of the four single chains and enzymatic proteolysis of each of the chains separately. These experiments must await the isolation in sufficient quantity of the four chains in chemically pure form.

SUMMARY

Carboxypeptidase acts on insulin, splitting off single amino acids in the first stages of the reaction. Certainly one, but possibly three of the terminal amino acids with a free carboxyl group of insulin consist of *alanine*. After 3 alanyl groups have been liberated, at least 6 amino acids appear almost or quite simultaneously.

Shortening the polypeptide chain of insulin by only a few amino acids results in the loss of the property to denature easily and in the loss of the biological activity.

RÉSUMÉ

La carboxypeptidase agit sur l'insuline en scindant au début de son action des acides aminés isolés. Certainement l'un, et peut-être trois, des acides terminaux de l'insuline présentant un groupe carbonyle libre, consistent en *alanine*. Après la libération de trois résidus alanyle, il apparaît, simultanément ou presque, au moins six autres acides aminés.

Un raccourcissement de quelques acides aminés seulement de la chaîne polypeptidique de l'insuline provoque la disparition de l'aptitude à la dénaturation et la perte de l'activité biologique.

ZUSAMMENFASSUNG

Carboxypeptidase wirkt auf Insulin indem sie in den ersten Stufen der Reaktion einzelne Aminosäuren abspaltet. Sicherlich eine, vielleicht aber auch 3 der endständigen Aminosäuren mit freier Carboxyl-Gruppe des Insulins bestehen aus Alanin. Nachdem 3 Alanylgruppen in Freiheit gesetzt worden sind, erscheinen mindestens 6 Aminosäuren gleichzeitig.

Wird die Polypeptidkette des Insulins nur um einige wenige Aminosäuren verkürzt, so verliert sie ihre leichte Denaturierbarkeit und ihre biologische Aktivität.

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