SOLID-PHASE EDMAN DEGRADATION OF A PROTEIN: N-TERMINAL SEQUENCE OF CYTOCHROME *c* FROM *CANDIDA KRUSEI*

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1. Introduction

Recently, the quantitative attachment of cytochrome *c* from horse heart to the *p*-phenylene diisothiocyanate (DITC) derivative of 3-aminopropyl glass (APG) as a support for solid-phase Edman degradation was demonstrated [1]. In the present study, cytochrome *c* from *Candida krusei* was used because it has a free α-amino group in contrast to cytochrome *c* from horse heart which is acetylated at its N-terminus. The *C. krusei* cytochrome *c* has been sequenced by Narita and Titani using conventional methods [2]. This protein was degraded by automated solid-phase Edman degradation over 35 steps with an average repetitive yield of 95%. In the course of this degradation we found that Glu in position 22 [2] actually in Gln. The results indicate that the scope of the solid-phase method might be extended to longer N-terminal sequences of proteins by the use of controlled-pore glass (CPG) derivatives as support and, in connection with this, by an optimized degradation cycle.

2. Experimental

2.1. *Attachment of cytochrome c to the p-phenylene diisothiocyanate derivative of 3-aminopropyl glass*

The DITC derivative of APG was prepared from CPG-10-75, 200-400 mesh, 75 Å mean pore diameter (Electro-Nucleonics, Inc., Fairfield, N.J., distributed by Serva, Heidelberg) as previously described [1]. 5.0 mg (400 nmoles) of native cytochrome *c* from *C. krusei* (Sankyo Company, Ltd., Tokyo) were dissolved in 0.3 ml H_2O, then 0.3 ml of pyridine were added. The red solution was shaken for 2 min with 200 mg of the dry support. The mixture was reacted with 0.1 ml of ethanolamine for 1 hr at room temperature. The loaded support was filtered, washed with methanol and dried in vacuo.

2.2. *Degradation in the automatic sequencer*

The degradation cycle of the automatic sequencer [1] is shown in fig. 1. Trifluoroacetic acid (TFA; Fluka, Neu-Ulm) was refluxed over CrO_3, distilled, dried over $CaSO_4$, and redistilled prior to use. Phenyl isothiocyanate (PITC; purissimum p.a., Fluka) was used purchased in a 10% solution in acetonitrile. The sequencing buffer was prepared according to Laursen [3]. Methanol (dried, p.a.), 1,2-dichloroethane (DCE; Uvasol) and acetonitrile (Uvasol) were used without further purification (Merck, Darmstadt).

2.3. *Identification of phenylthiohydantoins*

After conversion and purification on Dowex 50 X 4 [3] the resulting phenylthiohydantoins (PTH's) were identified either by gas chromatography (Phe, Ser, Ala, Val, Gly, Thr, Pro, Ile, Leu) or by thin layer chromatography (Glu, Gln). Arg and His were not identified. Gas chromatography was performed on a Beckman GC 45 gas chromatograph following the procedures of Pisano et al. [4] or Bober [5]. Thin layer chromatography was done on silica gel plastic sheets with a fluorescent indicator (F 1500 LS 254, Schleicher & Schüll, Dassel) in the solvents V and IV described by Jeppsson and Sjöquist [6].

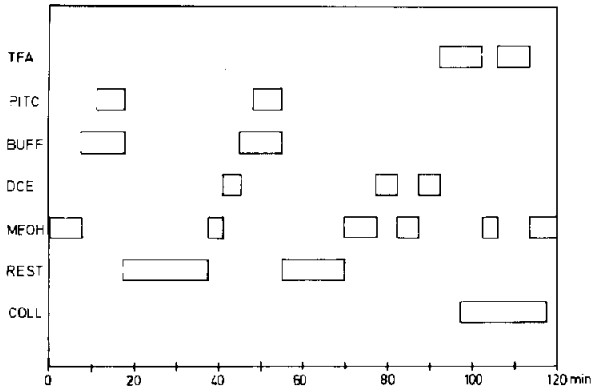

Fig. 1. Sequencer program for one degradation cycle in a 0.3 × 5 cm reaction column at 45°C: TFA, trifluoroacetic acid; PITC, phenyl isothiocyanate (10% in acetonitrile); BUFF, sequencing buffer [3]; DCE, 1,2-dichloroethane; MEOH, methanol; REST, no flow through the reaction column; COLL, phase of sample collection. Flow rates are 0.34 ml/min for TFA, PITC and BUFF; 0.65 ml/min for DCE and MEOH.

3. Results and discussion

As expected from previous experiments with cytochrome *c* from horse heart [1], the attachment of cytochrome *c* from *C. krusei* was completed within a few minutes, as monitored by the decrease of absorbance at 405 nm in the supernatant. It is essential to block excess isothiocyanate groups of the support with a primary amine. Ethanolamine was chosen, first for its convenient pK_a (9.5; [7]), and second for its hydrophilic character. The column pressure increases considerably when the reactive groups are not blocked.

For sequencing this protein, the degradation cycle was modified (fig. 1). Buffer–PITC phases were prolonged to a total of 47.5 min, interrupted by a methanol–DCE wash, to ensure a complete couplind reaction. TFA phases were shortened to minimize cleavage of peptide bonds yet made sufficiently long to achieve equilibrium in the reaction column. This condition could easily be deduced from the pressure profiles which are recorded routinely [1].

The degradation was carried out over 35 steps. Fig. 2 shows the gas chromatograms. Glu^5, Gln^6, Gln^{22} and Glu^{27} were identified unambiguously by thin layer chromatography. The only difference to the sequence presented by Narita and Titani [2] was

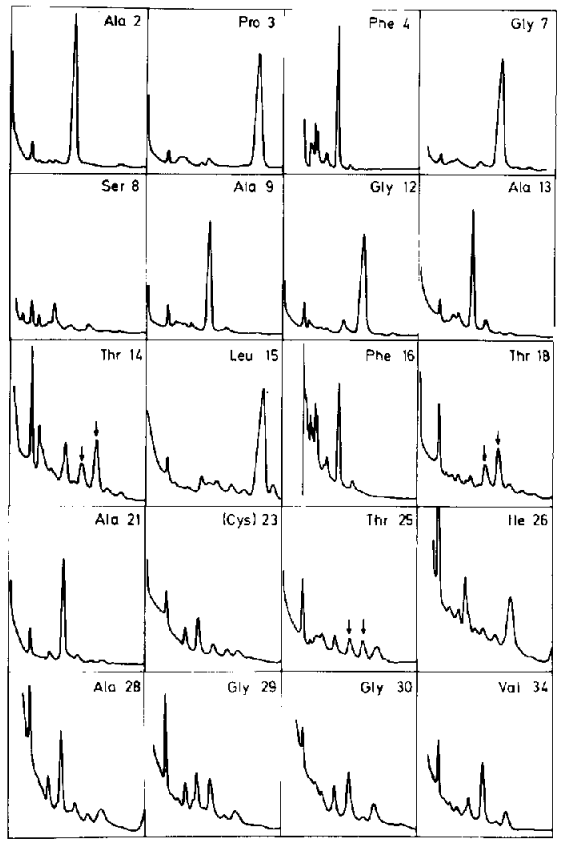

Fig. 2. Gas chromatograms of PTH's from the degradation of cytochrome *c* from *Candida krusei*. Sensitivities are increased 2-fold for steps 7, 8, 12, 14, 15 and 21, 4-fold for step 18 and steps 23–34 when compared to step 1. Arrows indicate the two peaks obtained for Thr PTH.

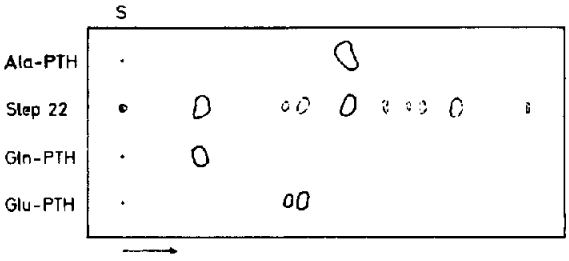

Fig. 3. Thin layer chromatography for identification of PTH from step 22. S: starting points. Standards: Ala PTH, Gln PTH and Glu PTH (Serva, Heidelberg) in methanol. For conditions see section 2.3. Ala PTH in step 22 is an overlap from Ala^{21}.

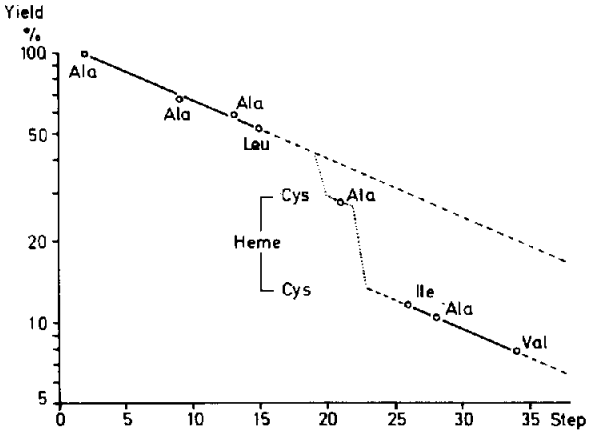

Fig. 4. PTH-yields from the degradation of cytochrome *c* from *Candida krusei* as estimated by gas chromatography. Yields in % of the yield in step 2. The dotted line is a hypothetical extrapolation for the decrease of yields in the heme region.

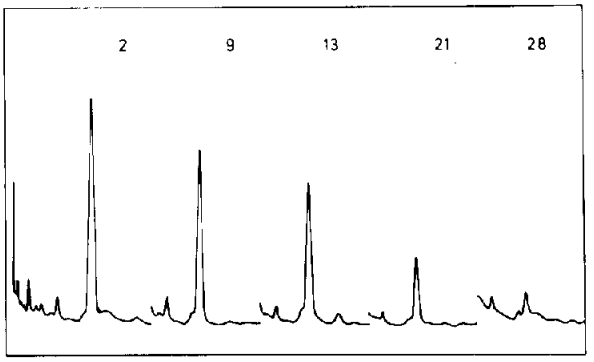

Fig. 5. Gas chromatograms of Ala PTH's from steps 2, 9, 13, 21 and 28. 1/20 of each sample was injected. Flame ionization detector, 6.4×10^{-10} A full scale; Ala^{28} PTH is about 2 nmoles.

a glutaminyl PTH in step 22 instead of a glutamyl PTH (fig. 3). This difference was reproduced in a second sequencer run.

Four hundred nmoles of cytochrome *c* attached to the support yielded 350 nmoles of alanyl PTH in the second degradation cycle. In the gas chromatogram of this step about 2% of the N-terminal proline are found. This proline should come from that small portion of the protein which is fixed solely by the ϵ-amino groups of lysines. In addition, the chromatograms reveal a low overlap until the heme-bound Cys^{20} is reached. The average repetitive yields calculated for the two sections from Ala^{2} to Leu^{15} and from Ile ^{26} to Val^{34} are equal and amount to 95%. Between these regions of identical repetitive yields (fig. 4) there is a drastic decrease of PTH yields in that part of the sequence which carries the heme (Cys^{20} to Cys^{23}). The persistance of the reddish brown colour of the glass after these steps indicates that the porphyrin ring or its degradation products remain fixed to the support. One may assume that in this part of the sequence the Edman reactions are sterically hindered. Without this hindrance it should be possible to identify at least 45 steps, extrapolating the equal repetitive yields before and after the heme region and assuming a limit of unambiguous identification of 10% of the starting yield (fig. 4). The signals of the alanyl PTH's from the positions 2, 9, 13, 21 and 28 in relation to the non-PTH background or to overlapping PTH's are shown in fig. 5. Since the complete attachment of cytochrome *c* has been proved, performic acid oxidation prior to coupling will provide further information about the maximum number of steps attainable in N-terminal sequencing of proteins by the solid-phase method.

It should be mentioned that there exists an optimal load for the DITC derivative of APG of about 1–2 nmoles protein/mg of support. In another experiment we attached 18 mg (1.45 μmoles) of cytochrome *c* to 200 mg of support. The sequencer run of this material produced a strong overlap of PTH's. We assume that less favourable sites of the support are occupied when the amount of protein is too high, and therefore the Edman reactions are not complete under the chosen degradation conditions.

Recently, we have also attached 3-phosphoglycerate kinase (EC 2.7.2.3) from yeast with a molecular weight of 47 000 [8] to the DITC derivative of an APG with 240 Å pore diameter. The high content of 46 lysines per molecule favours the attachment of this protein. For proteins with few or unaccessible lysines, aminoethylation of the cysteine residues might be helpful [9]. The higher molecular weight of proteins appears to constitute no principal limitation for the solid-phase method.

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