USE OF CARBOXYPEPTIDASE Y FOR CARBOXY – TERMINAL SEQUENCE DETERMINATION IN PROTEINS

by

B. MARTIN

I. SVENDSEN

and

M. OTTESEN

Department of Chemistry – Carlsberg Laboratory
Gamle Carlsberg Vej 10 – DK-2500 Copenhagen, Valby

Key words: Carboxypeptidase Y, sequence determination, proteins, sodium dodecyl sulphate

Treatment of several proteins-native pancreatic ribonuclease, human carbonic anhydrase B and human carbonic anhydrase C- with carboxypeptidase Y in the presence of 0.5% sodium dodecyl sulphate resulted in the sequential release of amino acid residues from the carboxyl terminus of the polypeptides. This provides a convenient method to study the carboxy-terminal sequence of proteins whose carboxyl termini are inaccessible in the native state to cleavage by carboxypeptidase.

1. INTRODUCTION

Compared with the carboxypeptidases of mammalian origin, carboxypeptidase Y isolated from baker's yeast has a much broader specificity including the ability to release proline residues (4). This makes it an excellent enzyme for use in the analysis of carboxy-terminal amino acid sequences in polypeptide substrates, as shown by HAYASHI et al. (4) and KUHN et al. (7). Unfortunately, in the case of native proteins this enzyme is normally unable to release any amino acid residues unless a denaturing agent is present. HAYASHI et al. (4) used 6 M-urea for this purpose and reported the sequential release of the first seven amino acids in good yields from ribonuclease. We have experienced difficulties with the use of this method, especially with respect to the separation of the small amounts of amino acids from the large excess of

urea present in the solution, and our yields of amino acids were consistently lower than expected.

It is the purpose of the present paper to describe an alternate procedure whereby urea is replaced by sodium dodecyl sulphate as the denaturing agent. Furthermore, it is demonstrated that this variation facilitates the recovery of the amino acids released by the carboxypeptidase Y during the incubation with pancreatic ribonuclease, human carbonic anhydrase B and human carbonic anhydrase C.

2. MATERIALS AND METHODS

Carboxypeptidase Y was isolated from Danish baker's yeast by the affinity chromatography procedure of JOHANSEN et al. (5). Ribonuclease A was a product of Sigma Chemical

Co., St. Louis, Mo., U. S. A. Human carbonic anhydrase B and C were prepared from human erythrocytes by affinity chromatography (6). Prior to use, the carbonic anhydrase isozymes were desalted by passage over a column of Sephadex G-25 (superfine, 1.0×40 cm) using H_2O as eluant and lyophilized. The carbonic anhydrase isozymes were stored at $-20^\circ C$.

Dowex 50 W X 12 (100-200 mesh) was a product of Fluka AG, Switzerland. Sephadex G-25 was a product of Pharmacia Fine Chemicals, Uppsala, Sweden. Sodium dodecyl sulphate and 2 [N-morpholino] ethane sulfonic acid were obtained from Sigma Chemical Co. All other chemicals were of analytical reagent grade and deionized water was used throughout.

The proteins were incubated, approximately 750 nmoles of protein, in 2-3 ml. of 0.1 M-2 [N-morpholino] ethane sulfonic acid buffer which was made 0.5% in sodium dodecyl sulphate (w/w) the pH of the solution being 6.30. Norleucine was added as a internal standard. Prior to the addition of the carboxypeptidase Y solution an aliquot was removed to serve as a blank. The release of amino acid residues was followed by taking aliquots from the reaction mixture at appropriate intervals. The reaction was terminated by transferring the aliquot to a test tube containing sufficient Dowex 50 W X 12 (H^+ -form, 100-200 mesh) to lower the pH of the supernatant upon shaking to between 2.2 and 2.7; e.g. approximately 1 ml wet resin per 100 μl aliquot. After twenty minutes, the resin was suspended in five times its own volume of water using a Vortex-shaker for thorough mixing. Upon settling of the resin, the supernatant was discarded and two further washes were carried out. The amino acids were eluted from the resin with 5 M- NH_3 according to the procedure of AMBLER (1). The resin was transferred to a 0.9×15 cm chromatography column and washed three times with 5 ml of 5 M- NH_3 each time. The NH_3 washes were pooled and the NH_3 removed by means of evaporation under reduced pressure. The residue was redissolved in 5 ml water and re-evaporated twice prior to amino acid analysis.

Analyses of the enzymatic digests were performed with a Durrum D-500 amino acid ana-

lyzer (range 1-10 nmoles per amino acid residue). All samples were diluted with pH 2.2 sodium citrate buffer (prepared according to the Durrum Instruction Manual) to an appropriate volume prior to introduction to the analyzer.

Protein concentration were determined spectrophotometrically using the published values of $E_{1\%}^{1\text{cm}}$ at 280 nm: ribonuclease 6.95 (3), carbonic anhydrase B 16.3 (9) and carbonic anhydrase C 18.7 (9).

3. RESULTS AND DISCUSSION

As previously demonstrated by HAYASHI *et al.* (4), carboxypeptidase Y was unable to release any amino acids from native pancreatic ribonuclease. However, we have found that carboxypeptidase was still active in the presence of 0.5% sodium dodecyl sulphate and in such a solution it sequentially released five carboxy-terminal amino acid residues-Phe-Asp-Ala-Ser-Val from ribonuclease (Fig. 1.). At a substrate

Figure 1. Rate of release of amino acids from ribonuclease A by digestion with carboxypeptidase Y. Protein concentration was $0.350 \mu\text{mole/ml}$ in a buffer containing 0.1 M-2 [N-morpholino] ethane sulfonic acid and 0.5% in sodium dodecyl sulphate (pH 6.3). The reaction was carried out at $22^\circ C$ with a substrate to enzyme ratio (mole/mole) of 1070:1.

The results are consistent with the sequence -Phe-Asp-Ala-Ser-Val (10).

Val ● Ser △ Ala ○ Asp ● Phe ○

Figure 2. Rate of release of amino acids from carbonic anhydrase B by digestion with carboxypeptidase Y. Protein concentration was 0.246 $\mu\text{mole/ml}$ in a buffer containing 0.1 M-2 [N-morpholino] ethane sulfonic acid and 0.5% in sodium dodecyl sulphate (pH 6.3). The reaction was performed at 22°C with a substrate to enzyme ratio (mole/mole) of 1200:1.

The results indicate the sequence -Thr-Val-Arg-Ala-Ser-Phe (2).

Phe $\square$ Ser $\triangle$ Ala $\circ$ Arg $\times$ Val $\bullet$ Thr $\bullet$

Figure 3. Rate of release of amino acids from carbonic anhydrase C by digestion with carboxypeptidase Y. Protein concentration was 0.256 $\mu\text{mole/ml}$ in a buffer containing 0.1 M-2 [N-morpholino] ethane sulfonic acid and 0.5% in sodium dodecyl sulphate (pH 6.3). The reaction was performed at 22°C with substrate to enzyme ratio (mole/mole) of 2265:1.

The results are in agreement with the sequence -Ala-Ser-Phe-Lys (8).

Lys $\times$ Phe $\circ$ Ser $\triangle$ Ala $\bullet$

to enzyme ratio of 1100:1 (mole/mole), the rate of release was quite rapid and similar to the release observed using 6 M urea as a denaturant as described by HAYASHI *et al.* (4). The yield of amino acids, on a mole to mole basis, was approaching 100% for the first two residues and was 75% for residue five.

The carboxy-terminal sequence of carbonic anhydrase B -Thr-Val-Arg-Ala-Ser-Phe (2) was also verified by the use of carboxypeptidase Y in the presence of 0.5% sodium dodecyl sulphate. The rate of release of amino acids from this substrate was fast with a substrate to enzyme ratio of 1200:1 (mole/mole) and it compared well with the rate observed for ribonuclease. The results show clearly the sequential release of the first six amino acids (Fig. 2) with yields for fully cleaved amino acid residues near 100%.

In the case of the carbonic anhydrase C, both the rate of release of carboxy-terminal residues and the number of residues released were lower. However, the results shown in Figure 3 did reveal the proper sequence -Ala-Ser-Phe-Lys for the first four residues (8) at a substrate to enzyme ratio of 2300:1 (mole/mole). The slow rate of release was in part due to the presence of a basic residue, L-lysine, in the carboxy-terminal position. The yield for the first two residues was only approximately 60%, but the reaction appeared to have levelled off. The low yield may perhaps be due to some blockage of the ϵ -amino group of the carboxy-terminal residue.

These results demonstrate the application of carboxypeptidase Y to sequence determination in the presence of sodium dodecyl sulphate to be a convenient method to use with protein substrates whose carboxyl termini would otherwise not be susceptible to cleavage.

ACKNOWLEDGEMENTS

One of us, B. M., would like to express his thanks for financial support to the Board of the Carlsberg Laboratory. We thank also cand. scient. JACK JOHANSEN both for helpful discussions and the carbonic anhydrase substrates and Professor T. VISWANATHA, University of Waterloo, Waterloo, Canada for valuable suggestions

during the early phases of this work. The technical assistance of BODIL CORNELIUSSEN with the amino acid analyses is also gratefully acknowledged.

REFERENCES

1. AMBLER, R. P.: Enzymic hydrolysis with carboxypeptidases. *Methods Enzymol.* 11, 155-166 (1967)
2. ANDERSON, B., P. O. NYMAN, and L. STRID: Amino acid sequence of human erythrocyte carbonic anhydrase B. *Biochem. Biophys. Res. Commun.* 48, 670-677 (1972)
3. BERNFIELD, M. R.: Ribonuclease and oligoribonucleotide synthesis. *J. Biol. Chem.* 240, 4753-4762 (1965)
4. HAYASHI, R., S. MOORE, and W. H. STEIN: Carboxypeptidase from yeast. *J. Biol. Chem.* 248, 2296-2302 (1973)
5. JOHANSEN, J. T., K. BREDDAM, and M. OTTESEN: Isolation of carboxypeptidase Y by affinity chromatography. *Carlsberg Res. Commun.* 41, 1-15 (1976)
6. JOHANSEN, J. T.: Isolation of human carbonic anhydrase B and C and apocarbonic anhydrase by affinity chromatography. *Carlsberg Res. Commun.* 41, 73-80 (1976)
7. KUHN, R. W., K. A. WALSH and H. NEURATH: Isolation and partial characterization of an acid carboxypeptidase from yeast. *Biochemistry* 13, 3871-3877 (1976)
8. LIN, K.-T. D. and H. F. DEUTSCH: Human carbonic anhydrase. The complete primary structure of the C isozyme. *J. Biol. Chem.* 249, 2329-2337 (1974)
9. NYMAN, P. O. and S. LINDSKOG: Amino acid composition of various forms of bovine and human erythrocyte carbonic anhydrase. *Biochim. Biophys. Acta* 85, 141-151 (1964)
10. SMYTH, D. G., W. H. STEIN and S. MOORE: Sequence of the amino acid residues in bovine pancreatic ribonuclease. Revisions and confirmations. *J. Biol. Chem.* 238, 227-234 (1963)