Carboxypeptidase Y from *Saccharomyces cerevisiae*: Circular dichroism and fluorescence studies

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Summary. Carboxypeptidase Y was isolated from *Saccharomyces cerevisiae* and its molecular structure investigated. The enzyme in the native state possesses 40% of its amino acid residues in a β-conformation. Its tryptophan residues seem to be largely buried in an apolar and unsymmetrical environment.

Acid carboxypeptidases are largely distributed in the plant kingdom as well as in yeasts[2]. They differ from carboxypeptidases of the pancreatic type in several respects. Indeed, they do not require divalent cations to be active, they are inhibited by diisopropyl phosphorofluoridate and display maximal activity in the acid pH range[3,4].

Carboxypeptidase Y isolated from *Saccharomyces cerevisiae* for the first time by Hata et al.[5] belong to the acid carboxypeptidase class[2]. This enzyme was recently shown to have a promising future in sequence works[2,6]. This is due to its ability to remove most of the amino acid residues including proline from the COOH termini of proteins and also to function quite well in denaturing media like 6 M urea. Furthermore, as a result of its amidase action, the enzyme might be applied to the sequence analysis of peptides having amidated COOH terminal groups such as oxytocin and vasopressin. Its active site is also well documented[4,7-13]. In this respect, carboxypeptidase Y seems to function quite similarly to chymotrypsin.

In *Saccharomyces cerevisiae* cells, the enzyme is located in the vacuoles while an inhibitor is found in the cytosolic fraction of the yeast. The physiological function of carboxypeptidase Y is however not yet known[14]. Also, little attention has been given, at the present time, to the molecular structure of the enzyme. It was the purpose of this communication to approach this question by means of circular dichroism and fluorescence studies.

Material and methods. Tryptophan (lot 41C-1520), N-benzoyl-L-tyrosine p-nitroanilide (lot 115C-5046), soybean trypsin inhibitor (lot 94-8190), bovine serum albumin (lot 37C-0440), bovine pancreatic ribonuclease (lot 56C-8020) and TRIS (lot 104C-5000) were purchased from Sigma. Serva provided guanidinium hydrochloride, a 1%-solution of which has an absorbance at 260 nm of less than 0.01; only freshly prepared solutions of this compound were used. Ampholines were obtained from LKB (Stockholm, Sweden). All the other reagents were of the best grade available.

Carboxypeptidase Y was prepared from *Saccharomyces cerevisiae* according to procedures described earlier[2,6]. In the course of purification, location of the enzyme in chromatographic eluates was performed by measurement at 410 nm of the product of the hydrolysis of N-benzoyl-L-tyrosine p-nitroanilide[15]. The purified enzyme was stored as a lyophilized powder at -20 °C. Disc gel electrophoresis on polyacrylamide (7.5%) were run at pH 9.5 and 4 °C according to the method of Ornstein[16] and Davis[17]. Protein bands were coloured with Coomassie Blue.

Isoelectric focusing experiments[18] were performed with the 110-ml LKB column. A linear pH gradient from 2.5 to 4.0 and a linear glycerol concentration from 0 to 60% were used. A potential of 500 V was applied during the experiment run at 4 °C for 48 h. The molecular weight was estimated by gel filtration on Sephadex G-75. The column (1×140 cm) was eluted at a flow rate of 10 ml/h with a 0.01 M phosphate buffer at pH 7.0. Fractions of 1.5 ml were collected. Bovine serum albumin, ribonuclease, ovalbumin and soybean trypsin inhibitor served as standards.

Protein hydrolysates, prepared by the method of Moore and Stein[19], were analyzed on a Durum amino acid analyzer. Tryptophan content was determined by the fluorimetric method of Pajot[20]. The carboxypeptidase Y concentration was determined spectrophotometrically with the use of a Zeiss PMQ II spectrophotometer. The value for $A^{1\%}_{1cm}$ at 280 nm of 15.0 was used in this work[2].

Fluorescence was measured at 25 °C with a Hitachi Perkin Elmer model MPF-2A spectrofluorimeter equipped with an Osram XBO 150-W Xenon lamp and a RCA 1P28 photomultiplier. A solution of L-tryptophan in water served as a standard. In order to correct for small instrumental fluctuations, this solution was recorded simultaneously with that of the protein solution. Excitation and emission bandwidths were 5 nm each. The protein concentration never exceeded 0.1 mg/ml. Under these conditions, fluorescence intensity could be linearly related to protein concentration. The circular dichroism curves were obtained with a Cary 61 spectropolarimeter. Measurements were made at 20 °C in quartz cells with a path length of 1 cm in the region above 250 nm and 1 mm below this wavelength. The optical density (path length of 1 cm) of protein solutions never exceeded 2.0 in the wavelength range used.

Results and discussion. Carboxypeptidase Y was isolated from *Saccharomyces cerevisiae* and purified according to Hayashi et al.[2] and Kuhn et al.[6] in 6 steps as follows: autolysis of the yeast cells, activation of the proteolytic enzymes, ammonium sulfate fractionation, 2 chromatographies on DEAE-cellulose and finally a chromatography on DEAE-Sephadex A-50.

The enzyme was shown to be homogeneous on disc gel electrophoresis at pH 9.5. The isoelectric point value: 3.3 obtained after isoelectric focusing experiments is in good agreement with the values 3.4[22] and 3.6[15] published earlier. Furthermore, the molecular weight we determined to be 69,000 daltons, lies between the values 61,000 and 75,000 daltons published by the same groups[15,22]. Finally, the amino acid composition of our preparation could not be distinguished from that published by Hayashi et al.[2]. In particular, the content in tryptophan residues determined after alkaline hydrolysis[2] or by the fluorimetric method of Pajot (this work) was found in each case to be 12 moles/mole of protein.

Parameters characterizing the fluorescence of carboxypeptidase Y from *Saccharomyces cerevisiae*

Properties	Tryptophan	Carboxypeptidase
λ_{max} emission	350-353 nm[a]	330 nm[b]
Band width	59-61 nm[a]	50 nm[b]
Quantum yield	0.20[d]	0.13[b]
K_Q (I^-)[c]	11.6 (M^{-1})	0.8 (M^{-1})
K_Q (NO_3^-)[c]	36.3 (M^{-1})	9.1 (M^{-1})
K_Q (Cs^+)[c]	1.9 (M^{-1})	0.0 (M^{-1})

[a] The free amino acid dissolved in water. [b] The protein was dissolved in 0.01 M phosphate buffer at pH 7.0. [c] The amino acid as well as the protein were dissolved in 2 mM HEPES buffer at pH 7.4. An excitation wavelength of 290 nm was used. [d] According to Brand et al.[21].

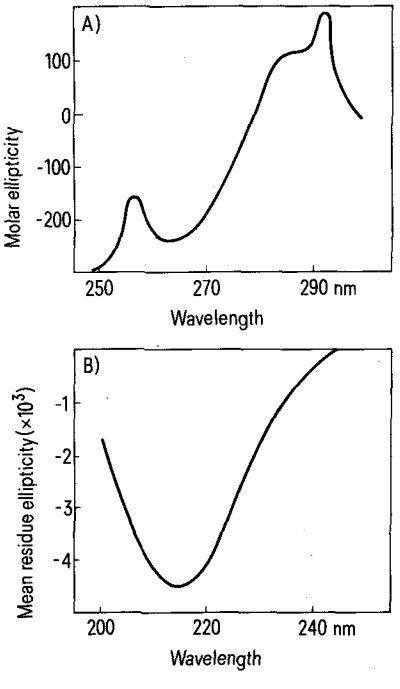
Circular dichroism studies. The circular dichroism curve of carboxypeptidase Y is shown in the figure. In the far UV-region (in the wavelength range from 200 to 250 nm), 1 minimum at 215 nm can be observed. Such characteristics have been obtained with poly-L-lysine in the β-conformation[23]. It corresponds also quite well to those spectra calculated for the regions of proteins with a β-structure[24]. In the spectrum of carboxypeptidase Y, no shoulder either at 207 nm or at 222 nm being apparent, it may be concluded to be due to the absence of any measurable α-helix structure in this protein. The latter thus seems be exclusively composed of a mixture of regions in a β-conformation and on the other hand of unorganized regions. Quantitatively, using the model of Chen et al.[24], the best fitting curve was obtained by assuming 40% of the amino acid residues folded in β-structure.

In the aromatic region (figure, A), several transitions are apparent; in particular 2 positive peaks respectively at 288 and 292.5 nm and 1 negative peak centered at 263 nm. The latter is possibly due to disulfide bonds present in the protein (carboxypeptidase Y possess 5 cystine residues). The amplitudes of the transitions in the region of tyrosine and tryptophan suggest an unsymmetrical environment for those residues.

Fluorescence studies. The parameters characterizing the fluorescence of carboxypeptidase Y are listed in the table. The band width, the quantum yield and the wavelength where the emission is maximal, are independent of the excitation wavelength between 275 and 300 nm. This observation strongly suggests that only 1 species of aromatic amino acid residue, namely tryptophan, is responsible for the measured fluorescence. The parameters cited however are a function of pH. The fluorescence intensity is maximal and constant for pHs between 6.0 and 8.5. On both sides of pH, it decreases. As a consequence, all subsequent measurements were performed at pH values around 7.

In proteins, as outlined by Burstein et al.[25], 3 classes of tryptophan residues can be identified by means of their fluorescence parameters. Class I, composed of tryptophan residues located at the surface of proteins in a polar (aqueous) environment, is characterized by a band width of 59–61 nm, a λ_{max} emission around 350 nm and a quantum yield of 0.20. Class II, composed of tryptophan residues partly buried and partly exposed to solvent, is characterized by a band width of 54–56 nm, a λ_{max} emission of 340–342 nm and a quantum yield of 0.30. Finally class III, composed of residues completely buried, in a apolar environment is characterized by a band width of 48–49 nm, a λ_{max} emission of 330–332 nm and a quantum yield of 0.11. Obviously, from the parameters listed in the table, the tryptophan residues of carboxypeptidase Y can be deduced to belong to class III.

To check this possibility, the study of the effect on tryptophan fluorescence of ionic quenchers was undertaken[25,26]. Cesium cations and iodide and nitrate anions were chosen for that purpose. The values of the Stern-Volmer constants (K_Q) obtained for tryptophan as the free amino acid and for carboxypeptidase Y are listed in the table. From these values accessibility percentage of 0% (Cs^+), 7% (I^-) and 25% (NO_3^-) can be estimated. The tryptophan residues of carboxypeptidase Y are so confirmed to be largely buried in the hydrophobic core of the protein.



Circular dichroism spectra of carboxypeptidase Y in the aromatic (A), and in the far UV-(B) regions. Protein concentration in 0.01 M phosphate buffer at pH 7.0 was 0.8 mg/ml. Results are given as mean residue ellipticity expressed in degrees × cm^2 per dmole of amino acid residue.

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