CARBOXYPEPTIDASEY FROM SACCHAROMYCES CEREVISIAE CONFORMATIONAL DIFFERENCES REFLECTED IN KINETIC BEHAVIOUR IN WATER AND DEUTERIUM OXIDE

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Abstract

The glycoenzyme carboxypeptidase Y (peptidyl-amino-acid hydrolase, EC 3.4.16.1), from baker's yeast (British Fermentation Products Strain, Ng 72), of motecular weight 60,000, and a protion closely similar to those in the literature for carboxypeptidace Y isolations from other yeast sources, but was 24.3 wt% ca bohydrate (mannose 83% by wt. with traces of galactose and galє osamine). Circular dichroic spectra indicated that the enzyme lost its β - structure as the pH was lowered from 8.08 to 4.16. At p^2H 8.22 in ^2H_2O media the conformation of this enzyme was different from that observed at pH 8.08. A tyrosine residue appeared to be perturbed by lowering the pH of the medium. Carboxypeptidase Y was rapidly, and essentially irreversibly, inactivated at low p^2H. The pH profile of k_{cat} for the carboxypeptidase Y-catalysed hydrolysis of 4-nitrophenyltrimethylacetate showed two inflections at 45°C: one at $pK_{app} \sim 3.7$ insensitive to temperature variation (ascribed to a carboxyl group), and one of $pK_{app} \sim 5.7$ markedly temperature-dependent and possibly caused by a histidine residue.

In the yeast life-cycle, protease action has been implicated in cell-budding [1], ascospore formation [2], tryptophan synthesis [3], as well as in the modification of several other enzyme activities [4]. Carboxypeptidase Y (EC 3.4.16.1) from yeast, useful in protein sequencing and related studies [5—7], is an intracellular protease. a group of enzymes into which mechanistic insight is sadly lacking.

For carboxypeptidase Y from Japanese yeast, the inhibition by L-phenylalanine changes on going from H_2O to ^2H_2O media in studies with Nacetyl -L-phenylalanyl ethyl ester [9]. We have suggested that the conformation or active-site configuration of carboxypeptidase Y differs from H_2O to ^2H_2O [8].

Carboxypeptidase Y (spec. act. > 100) was prepared from British Fermentation Products (strain Ng 72) compressed baker's yeast by affinity chromatography, [11] followed by G-75 gel filtration. Approximate enzyme concentrations were estimated by using $A_{280}^{1\%}$=15 and a molecular weight of 61000 [10, 12]. Accurate functional enzyme concentrations were determined by active-site titration [10]. Activity measurements and kinetic procedures [8,14] and amino acid and carbohydrate analyses were effected as described [10, 13]. The carboxypeptidase Y preparation was homogeneous by SDS-polyacrylamide gel electrophoresis with molecular weight 60 000 and 55 000 on separate occasions with different preparations. Homogeneity was also indicated by the sedimentation velocity experiment with sedimentation coefficient 3.98 S in 0.01 M phosphate buffer. Values of s_{20}^0 = 4.41 S for the enzyme from Danish yeast [11] and $s_{20.}^0$ w = 4.3 S for that from Japanese yeast [12] have been reported. The carbohydrate content was 25.3 wt%; the molecular weight of the carbohydrate protion was 15.18. 10^3 based on mo based on molecular weight of the enzyme as 60. 10^3.

Small amounts of galactosamine (0.1%), galactose (0.8%) and glucose (0.6%) were found in addition to mannose (21.2%) and glucosamine (2.6%). Mannose accounted for 83.8% by weight of the total hexoses, an glucosamine 10.3%. Amino acid compositions were similar to those previously reported [10]. The total number of residues calculated was 382 (cysteine and tryptophan not determined) and the estimated

molecular weight was 41.10^3 for the protein portion.

CD spectra of carboxypeptidase Y in H_2O and ^2H_2O at corresponding pH and p^2H values showed significant differences. In the near-ultraviolet region, pH (or p^2H)-dependent perturbation of side-chain conformation was observed (Figs. 1 and 2), with the degree of perturbtion greater in ^2H_2O than H_2O. Values of $[\theta\;]$ (280–300nm) at p^2H 8.22 were positive (+10 to +15 deg. cm^2. $dmol^{-1}$), but became negative as the p^2H was decreased. At p^2H 4.13 a negative trough around 290–300 nm became significant, which may indicate perturbation of tryptophan (s). The negative band at 280 nm may be caused by perturbation of tyrosine(s). At pH 4.16 such perturbations were considerably less marked.
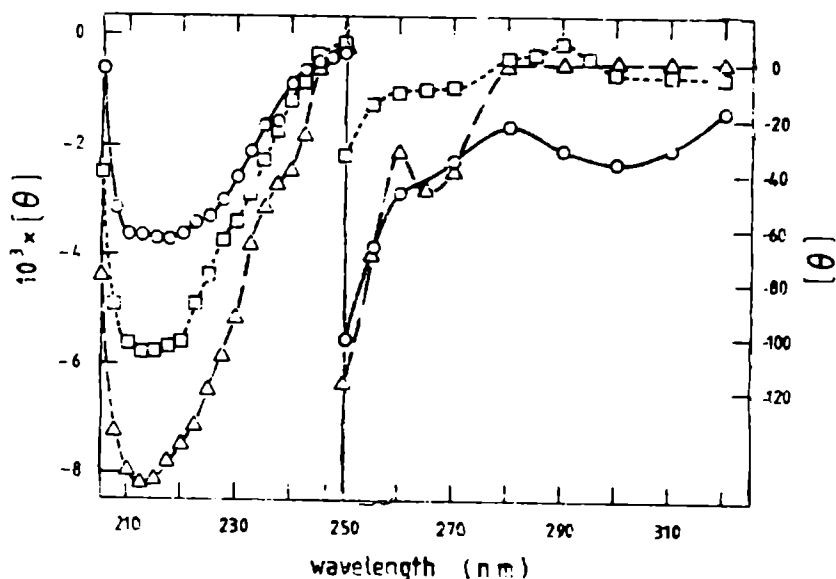

Fig. 1. Dependance on pH of CD spectra for carboxypeptidase Y in ^1H_2O media; conditionsdescribed in text. CD spectra were measured using a Cary 60 spectro-polarimeter with a 6001 CD accessory. Fused quartz cells with 10 mm (250 to 300 nm) and 1 mm (200 to 250 nm) pathlength were used. Mean residue ellipitcity. [0], was calculated using a mean reiduce weight of 107 for the enzyme. Average dichroic absorbarces were determined from triplicate recordings. O, pH 4.16; pH 6.90;   , pH 8.08.

In the far-ultraviolet region the effects in ^2H_2O were more pronounced than in H_2O. In H_2O media at pH 8.08 or 6.90 the enzyme showed β -structure, but lost its structure and became more disordered at pH 4.16. In ^2H_2O media the enzyme was considerably disordered even at p^2H 8.22; the degree of disorder increased as the p^2H decreased further. Comparing pH 8.08 and p^2H 8.22, the enzyme is less ordered in the ^2H_2O medium.

The activity pH profile in H_2O of British enzyme (Fig. 3) is similar to that in ^2H_2O of Anheuser-Busch enzyme [8]. The ionisation at lower PH for British enzyme had $pK_{app} \sim$ 3.7, at both 25 and 45°C, and the enzyme, once subjected to treatment with low pH buffer, even in the presence of substrate, is inactivated essentially irreversibly. Thus
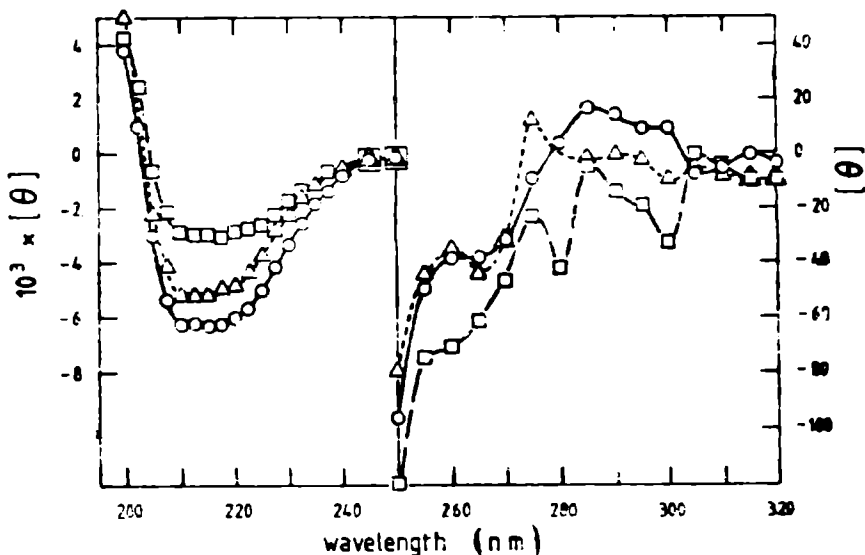

Fig. 2. Dependance on p^2H of CD spectra for carboxypeptidase Y in ^2H_2O media , p^2H 4.13; , p^2H 6.87; O, p^2H 8.22.

the mactivation (pK_{app} 3.7) in H_2O for the British enzyme is analogous to that observed for the Anheuser-Busch enzyme in ^2H_2O [8]. However, the British enzyme loses activity very rapidly at lower pH. For peptide sequencing studies using carboxypeptidase Y from British yeast acidic conditions must be avoided. The temperature insensitivity [15] of the group of $pK_{app} \sim 3.7$ controlling the inactivation process suggests that it might be a carboxyl group.

The group of higher pK_{app} is possibly involved in the catalytic mechanism, either directly by being situated close to the active site, or through control of the active-site configuration. The pK_{app} value appears to increase from 5.7 at 45°C to more than 7 at 25°C, implying that $\Delta H_{ionisation}$ is positive. The positive value of $\Delta H_{ionisation}$ and pK_{app} range of $5.7 -> 7$ leads us to suggest tentatively a histidine residue. As the pH is dropped to 4 much of the β-structure of the British enzyme is lost, a feature presumably reflected in the rapid deactivation of the enzyme at lower pH values. More remarkable, perhaps, is the fact that the enzyme β-structure is strongly disordered at $p2H$ 8. This difference in secondary structures at pH8 and p^2H8 would explain the observations of Bai et al. [9], who reported that inhibition kinetics of Lphenylalanine versus N-acetyl-L-phenylalanyl ethylester as substrate changed from competitive in H_2O at pH 6.5 to non-competitive in ^2H_2O (p^2H 7.0). The CD results on the British enzyme directly support the view of a conformational difference between ^2H_2O and H_2O.
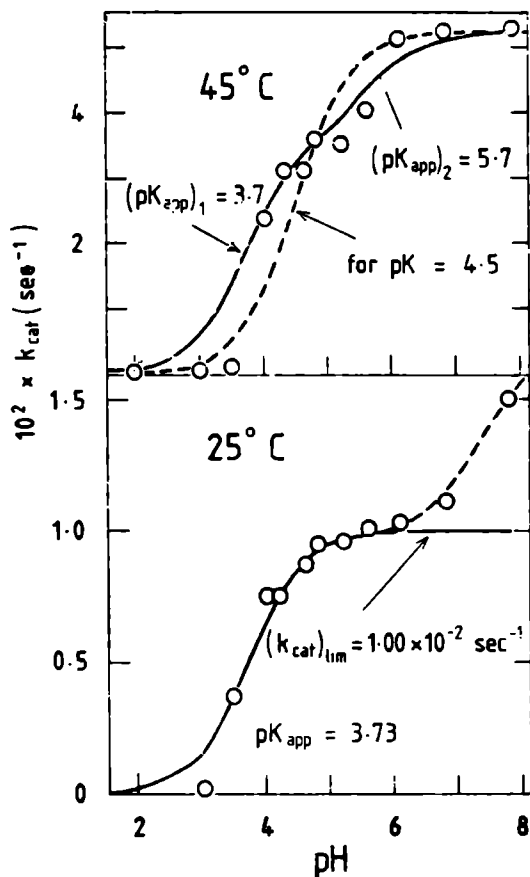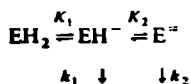
Fig. 3. Profiles of Ph dependences of K_{cat} at 45 and 25°C for the carboxypeptidase Y - catalyzed hydrolysis of 4 - nitrophenyltrimethy - lacetate in aqyeous (^1H_2O) media. For the 45°C figure (upper) the points are experimental and the solid line is theotrtical for two indendent ionisations of pK_{app} 3.7 and 5.7, respectively, for the kinetic scheme

$$EH_2 \overset{K_1}{=\!=} EH^- \overset{K_2}{=\!=} E^=$$
$$k_1 \downarrow \qquad \downarrow k_2$$

where $K_1 = 3.6.10^{-2}$ s^{-1} and $k/2 = 5.2.10^{-2}$ s^{-1}. For comparison the dotted the line describes a single ionisation of pK = 4.5 with a limiting value of $K_{eat} = 5.2.10^{-2}$ s^{-1}. The lower figure represents the same reaction as described above, but at 25°C. The points are experimental and the solid line is theoretical for an ionisation of $pK_{app} = 3.73$ with a limiting value of $K_{cat} = 1.00.10^{-2}$ s^{-1}. The dotted line is notional to assist visualisation thath the higher pH values.

34

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