Cytochrome c Peroxidase

IV. Isoelectric Focusing and Analysis of the Crystalline Enzyme in an Acidic pH Gradient

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Crystalline cytochrome *c* peroxidase (CCP) was found to be homogeneous in moving boundary electrophoresis and to have an isoelectric point equal to 4.9 in an acetate buffer, $\mu = 0.1$, at 4°C.[1] However, horse radish peroxidase and lactoperoxidase have both been found to consist of several fractions and it seems that this is a general feature of peroxidases.[2-6] In order to elucidate the possible existence of such subfractions in crystalline preparations of CCP, these were studied with the electrofocusing technique of Svensson and Vesterberg.[7,8] The results of these studies are reported in this communication.

Material and methods. Crystalline CCP ($A_{407}/A_{280} = 1.29$) was prepared from baker's yeast and the enzyme assayed as described previously.[1] Several preparations were studied, of which two are chosen to represent different patterns of heterogeneity. Preparation I was freshly isolated and care was taken to avoid all degrading conditions as far as possible, and preparation II had been stored for 12 months at −16°C.

Isoelectric focusing was carried out in an electrofocusing column of type LKB 8101, having a capacity of 110 ml. The instructions of the manufacturer were essentially followed. The density gradient was effected with sucrose (0−50 % w/v). The carrier ampholytes forming the pH gradient were supplied by LKB-Produkter AB, Stockholm; they covered the pH range 4−6 and were used at a concentration of 1 % (w/v). The CCP sample (about 5 mg) was dialyzed against 1 % glycine and mixed with one of the middle fractions of the gradient. 0.2 ml of concentrated phosphoric acid was added at the anode and 0.2 ml of ethylenediamine was added at the cathode. The column was held at +3°C. Electrophoresis was initially performed for 3.5 h at 500 V and subsequently for 20 h at 700 V. When the proteins had been focused, the column was drained and 1 ml fractions were collected.

Spectra were recorded with a Beckman DU-2 spectrophotometer and a Beckman DK-1A recording spectrophotometer. Before the determination of the ratio A_{407}/A_{280}, the protein solutions were dialyzed against distilled water overnight.

The pH of the effluent was measured with a Radiometer PHM 4c pH meter and a conventional combination (glass-calomel) electrode. The pH meter was calibrated with a phthalate buffer. All of the measurements were performed at +3°C.

Results. Application of the isoelectric separation technique in an acidic pH gradient revealed that the different CCP preparations contained one main component and several subunits, whose number and proportions varied. Fig. 1 shows a

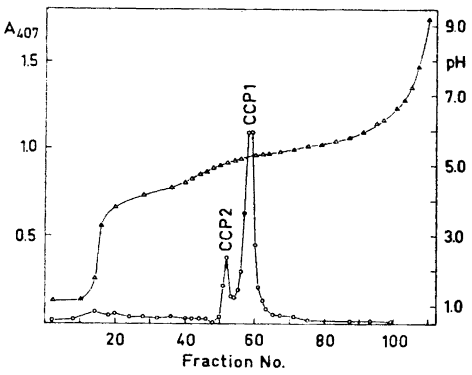

Fig. 1. Fractionation of crystalline CCP (preparation I) from baker's yeast by electrofocusing. The experimental conditions are described in the text. ○ absorbance at 407 nm, △ pH.

typical focusing pattern of a highly purified crystalline CCP (I). The technique could be used to separate the components in quantities sufficient for chemical characterization. The ratio A_{407}/A_{280} for the two components was measured after dialysis and found to be higher than before separation (CCP 1 = 1.35 and CCP 2 = 1.30). The spectra of the two components were identical with the spectrum of crystalline CCP. The isoelectric points of the two components differ slightly: 5.21 and 5.10 as measured at +3°C.

Another focusing experiment (Fig. 2) with another crystalline preparation (II)

of CCP revealed four components of which two evidently are identical with CCP 1 and CCP 2 of Fig. 1. The concentration of CCP 2 in this preparation was significantly higher than that of CCP 1. The opposite is

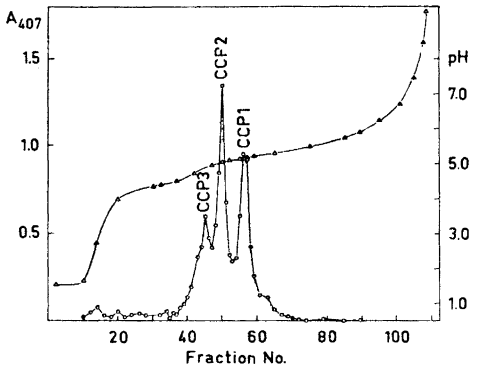

Fig. 2. Fractionation of crystalline CCP (preparation II) from baker's yeast by electrofocusing. The experimental conditions are described in the text. ○ absorbance at 407 nm, △ pH.

the case for the preparation of Fig. 1. The two components gave the same spectrum and had the same specific activity as the original crystalline CCP. Their isoelectric points were 5.17 and 5.09 and their ratios of A_{407}/A_{280} 1.35 and 1.30, respectively. Subfraction CCP 3 revealed a double band on the column under steady state conditions. It was, however, impossible to determine the exact pI's of CCP 3_I and CCP 3_{II} since these components could not be identified by spectrophotometric measurements on fractions that emerged from the column. However, the difference in pI between CCP 3_I and CCP 3_{II} was estimated to be only 0.03—0.04 pH units from the pH gradient in this region. A mixture of these two fractions was enzymatically active and gave a spectrum identical with that of the original crystalline CCP preparation.

Discussion. These results suggest that CCP 1 can be converted into CCP 2. It seems that the CCP 1 molecule contains one or more labile amide groups which partially hydrolyze even under the mild conditions during purification. If so, CCP 1 would correspond to the fully amidated molecule that forms CCP 2 by releasing one labile group. The difference of about 0.1 pH units in the isoelectric points of CCP 1 and CCP 2 could well reflect a difference of one unit charge between the two components.

As the isoelectric points of CCP 3_I and CCP 3_{II} differ very little, it is reasonable to assume that the number of amide groups is the same in both components. The difference of 0.03—0.04 pH units between these two variants may be explained by the liberation of carboxyl groups with different pK_3' values. For example, the pK_3' value of the β-carboxyl group of aspartic acid is equal to 3.65 and that of the γ-carboxyl group of glutamic acid equal to 4.25.[9]

The results of the experiments described in this communication support the view that subfraction CCP 2 arises by conversion from the main fraction CCP 1 and subfraction CCP 3 from CCP 2, since subfraction CCP 3 was observed in preparations with a high content of CCP 2. The extent to which these subfractions are present in intact yeast cannot be decided on the basis of these experiments.

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