ELECTRON MICROSCOPY OF PIG LIVER CARBOXYLESTERASE

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1. Introduction

During the past decade high-resolution electron microscopy has become increasingly important as an additional tool for elucidating the subunit structure of many enzymes [1]. In the present paper we wish to report an electron microscopic study of pig liver carboxylesterase (EC 3.1.1.1). This enzyme is a dissociating protein with a molecular weight of 163 000–168 000 as was found independently in three laboratories [2–4]; its subunit composition, however, is still controversial [5, 6].

2. Methods

Carboxylesterase was isolated from pig liver microsomes as described previously [7]. In addition, the enzyme was subjected to a final gel filtration on Sephadex G-200. The preparation was diluted to a concentration of about 0.4 mg/ml with 0.005 M Tris-HCl buffer, pH 8.6, containing 0.001 M ethylene diamine tetraacetic acid. The enzyme preparation used in this study had a specific activity of 380 IU/mg protein (substrate 0.05 M methyl butyrate; t = 30°; pH 8.0; pH-stat technique [8]). Crystalline catalase from bovine liver (for analytical purposes) was purchased from C.F. Boehringer u. Soehne/Mannheim.

The electron microscopic grids were prepared by evaporation of carbon onto "Zaponlack"-coated grids by a Speedivac High Vacuum Coating Unit (Edwards Ltd, Sussex, England). They were cleaned by ionic bombardment and subsequent washing in chloroform. The enzyme was mixed with an equal part of Tris buffer, pH 8.0, containing 2% glutaraldehyde and 10% glycerol. After about 2 min one drop of this mixture was applied to the grid which was placed on a filter paper. Negative staining was performed by adding one drop of 10% unbuffered ammonium molybdate pH 6.5.

The grids were examined in a Philips EM 300 electron microscope at a magnification of 201 100. The objective diaphragm had an aperture of 20 μ, the accelerating voltage was 80 kV.

3. Results

It was observed in preliminary experiments that neutralized phosphotungstic acid, the reagent most frequently used for negative staining of proteins, appeared to effect a dissociation of pig liver esterase and only monomeric particles could be detected following its application. In contrast, associated oligomeric forms became visible when pig liver esterase was subjected to negative staining by ammonium molybdate. Another problem was the tendency of the enzyme to form clots or unresolvable aggregates on prolonged standing. These aggregates did not lead to the formation of visible precipitates and could be seen only by electron microscopy. For this reason pig liver esterase should be examined immediately after the last step of the isolation procedure.

The structure of negatively stained pig liver ester-

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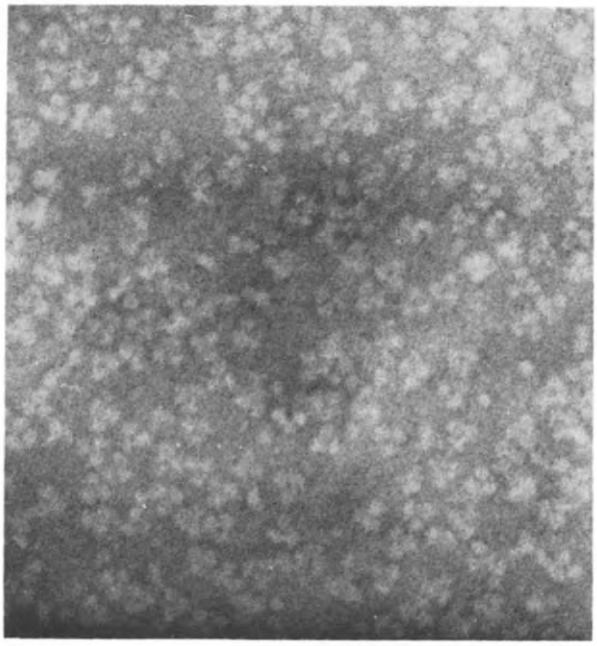

Fig. 1. Pig liver esterase, negatively stained with ammonium molybdate. Magnification × 374 000.

ase is shown in fig. 1. Most of the molecules seem to consist of three subunits arranged in a clover-leaf shape. Between the subunits there is a darker y-like slot and a dot in the middle. In addition, several single particles can be seen, whereas dimeric structures are rare. A few forms have the appearance of tetramers. Independent counting of 380 particles of the same preparation by three persons gave the following distribution: apparent monomers 13.6% (9.7–16.0); dimers 9.6% (6.0–12.1); trimers 59.4% (56.7–62.4); tetramers 5.7% (2.0–8.7); doubtful forms 11.8% (4.3–17.9). The values represent the mean, the highest and lowest figures being given in parentheses. Thus, the individual deviations in the counting results are relatively small.

In order to estimate whether the dimensions of the particles as seen by electron microscopy are in reasonable agreement with the results of subunit weight determinations by other methods [5], it was assumed i) that the particles are spherical and ii) that the partial specific volume of the enzyme ($\bar{v}$ = 0.738 ml/g [2]) is not altered by the negative staining procedure. The mean of 25 measurements of particle radius was 26.65 ± 1.35 Å. Using the formula

$$\text{mol. wt.} = \frac{4\,r^3 \times N}{3\bar{v}}$$

(where r = mean radius, N = Avogadro's number and $\bar{v}$ = partial specific volume), this corresponds to a particle weight of 64 500. If the same procedure was applied to negatively stained catalase a value for r of 26.86 ± 3.78 Å per subunit was obtained, from which the subunit weight of this enzyme was estimated to be 66 100. When negatively stained bovine liver esterase was examined at a concentration of 0.4 mg/ml, only monomeric particles could be detected by electron microscopy. This is in agreement with the previous observation that ox liver esterase dissociates more readily, i.e. at higher protein concentrations, than pig liver esterase [5, 9].

4. Discussion

It has been previously reported that pig liver esterase (mol. wt. 163 000–168 000 [2–4]), reversibly dissociates under suitable conditions into active half-molecules with molecular weights between 75 000 and 90 000 as concluded from ultracentrifugation and gel filtration studies [3, 4]. In a recent paper from this laboratory, however, a trimeric structure was proposed, based on the results of disc electrophoresis experiments in the presence of sodium dodecyl sulfate, on gel filtration in 6 M guanidine and on quantitative determination of the N-terminal groups [5]. All three methods yielded subunit weights in the range of 55 000–63 000. In SDS-disc electrophoresis one sharp band only was found, indicating that the subunits are identical with respect to size.

As shown in fig. 1 the electron microscopic examination of negatively stained pig liver esterase under the conditions specified revealed that about 60% of the particles appear as clover-leaf shaped trimers. About 10% seem to be dimers. Of course, these may either be true dimers or actually trimers as seen from a lateral position. About 6% of the particles could be interpreted as tetramers, though these could conceivably be formed by fortuitous addition of a single particle to a trimer. It is feasible, however, that the

percentage of tetramers is considerably higher, if one assumes that some or all of the particles which are apparently trimers could actually be tetramers in which the four spherical subunits form a tetrahedron and that the tetrahedrons are oriented on the film point downwards and base upwards. This possibility, however, is not considered to be very likely.

In addition, the rough calculation of the subunits weight from the electron microscopic measurements gives a value (64 500) quite close to the known subunit weight [5]. It is, however, about 10–15% higher. One of the many possible reasons for this is that the assumption of the subunits being spheres is a probably erroneous simplification. If the particles are not completely in focus, they would also appear somewhat too large. In any case, the calculated subunit weight is more in favour of a trimer than of a tetramer. It might be mentioned here that analogous calculations of particle weights from their electron microscopically determined dimensions have given good or even excellent agreement with the physical data in the case of several other enzymes [10–12].

As a control we have calculated in the same way the subunit weight of beef liver catalase which is very similar to that of pig liver esterase [5]. In addition, catalase is a well-characterized enzyme for which detailed ultracentrifugation data on its molecular and subunit weight as well as electron microscopic observations are available [13]. In the case of catalase the tetrameric structure is easily visible under the electron microscope [13, 14]. The radius of the subunit (26.86 Å) and the correspondingly calculated subunit weight are slightly larger than in pig liver esterase. The particle weight of 66 100 as calculated from the electron microscopic picture is also about 10% higher than the accepted "biochemical" value. Thus, the results of our electron microscopic observations can be interpreted as further evidence that the subunit size of beef liver catalase and pig liver esterase is about the same.

In conclusion, the results of the present electron microscopic study lend further support to the proposal that pig liver esterase at a concentration of 0.4 mg/ml and at pH 8.6 exists mainly in trimeric form. Of course, the equilibrium between monomer, dimer and trimer depends on the experimental conditions, such as protein concentration, pH, and salt concentration [4, 6]. Although the concept of a trimeric protein seems to be rather unusual, a recent compilation of the subunit structure of numerous proteins shows that there are at present at least 9 proteins reported to be composed of three subunits [15–17]. Our results are quite inconsistent with a dimeric structure for pig liver esterase, whereas the possibility of a tetrahedral tetramer cannot be completely ruled out.

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