THE EQUIVALENT WEIGHT OF PIG LIVER CARBOXYLESTERASE (EC 3.1.1.1) AND THE ESTERASE CONTENT OF MICROSOMES

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Received 10 November 1974

1. Introduction

In recent publications it has been shown that pig liver carboxylesterase [1–4] and also esterases from other tissues [1,4–6] consist of three subunits of an approx. mol. wt of 60 000. These findings are inconsistent with earlier results obtained by titration with organophosphorus inhibitors [7–9] which indicated that pig liver esterase contains two active sites per molecule, i.e. one mole of active site per about 80 000 g of protein. In this communication we investigate possible reasons for this discrepancy. If one assumes that numerical values of about 80 000 are too high for the equiv. wt, this might be accounted for on the following grounds: 1) the preparations of esterase used were not pure; 2) the preparations were pure with respect to proteinchemical criteria, but contained partially denatured molecules not reacting with the organophosphate inhibitor; 3) the methods of protein estimation used were calibrated incorrectly. In order to exclude the possibilities 1 and 2 we purified a labeled esterase from microsomes pretreated with the radioactive inhibitor bis-4-nitrophenylphosphate (BNPP-^{14}C) BNPP is bound covalently, specifically, and irreversibly to the active site op pig liver esterase [10]. Hence, the equiv. wt can be obtained from the specific radioactivity at each step of the isolation procedure. Partial denaturation of the previously labeled enzyme during the subsequent isolation procedure would not alter the specific radioactivity. Possibility 3 might be ruled out by careful re-examination of the accuracy of the methods for protein estimation.

Abbreviations: BNPP: bis-4-nitrophenyl-phosphate; E600: 4-nitrophenyl-diethyl-phosphate; SDS: sodium-dodecyl-sulfate.

If carboxylesterase is the only protein in microsomes which binds BNPP-^{14}C, the esterase content of microsomes may be calculated from the specific radioactivity of the labeled microsomes.

2. Materials and methods

BNPP-^{14}C was synthesized by Farbwerke Hoechst, Frankfurt GFR. Its radiochemical purity was tested by thin-layer chromatography and scanning with the LB 2723 scanner (Berthold and Friesede, Wildbad GFR). We got a single radioactive spot with the following solvents (W. Block, personal communication): methanol-dichloromethane–10% NH_3 = 20:80:3 (v/v), R_f = 0.24; butanol-acetic acid–water = 4:1:1 (v/v), R_f = 0.62; isopropanol–25% NH_3 = 8:2 (v/v), R_f = 0.68; methanol-dichloromethane-acetic acid = 20:75:5 (v/v), R_f = 0.27.

Sephadex was purchased from Pharmacia, Frankfurt GFR, and Ampholine from Colora, Lorch GFR. E 600 was kindly provided to us by Bayer, Leverkusen GFR. The two methods for the isolation of pig liver microsomes and carboxylesterase were published recently [11].

The following methods were used for the estimation of protein concentration: nitrogen was determined according to Kjeldahl with the apparatus described by Klingmüller et al. [12]; 'Selenreaktionsgemisch' (Merck, Darmstadt GFR) served as catalyst, and the boric acid was titrated with an autotitrator (Radiometer, Copenhagen, Denmark). A micromodification [13] of the biuret method [14] was employed in which the turbidity was eliminated by means of KCN [15].

$A_{280\ nm}$ was measured in quartz cuvettes with the

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PMQ II spectrophotometer (Zeiss, Oberkochen GFR). The radioactivity of liquid samples was measured with a dioxan/naphthaline scintillator in the Tricarb liquid scintillation counter (Model 3380, Packard, USA). Disc gels were cut into about 1 mm segments, and these were homogenized with 0.5 ml of water in a glass homogenizer with a teflon pestle. The homogenates were tested for radioactivity as described above. The specific radioactivity (R_0) of standard solutions of BNPP-^{14}C was measured as follows: 0.1 ml of standard solution was mixed with 1.9 ml of 0.1 M phosphate buffer pH 8.0, and A_{405} was measured (A_1). Another 0.1 ml aliquot of the standard solution was hydrolyzed for 3 hr at 100°C with 1.9 ml of 1 N NaOH in a sealed vial, then neutralized with HCl, and made up to 5 ml with pH 8.0 buffer before measuring its A_{280} (A_2). If N means counts per l of standard solution,

$$R_0 = \frac{N \cdot 16440}{25A_2 - 20A_1} \qquad \left(\frac{\text{counts}}{\text{mole of BNPP}}\right)$$

A_1 was assumed to be due to traces of free 4-nitrophenol, it was nearly negligible because of the high radiochemical purity of the BNPP used.

The equiv. wt of non-labeled esterase was estimated with E 600 according to Krisch [7]. We employed double chamber cuvettes (2 × 2 cm light path) with 0.1 M phosphate buffer pH 8 and 2×10^{-4} M E 600 in chamber a 1, buffer and enzyme in chamber a 2, buffer and water in chamber b 1, and buffer, E 600, and enzyme in chamber b 2. The initial burst (A_{405}) occurring in chamber b 2 was measured after 3 min at 25°C. A second value could be read after adding E 600 to chambers a 2 and b 1. A molar absorptivity (405 nm) of 16440 [10] was used for calculation.

3. Results

3.1. *Determination of the esterase contents of microsomes*

2 ml aliquots of pig liver microsomes in pH 8.5 [11] buffer were reacted with a solution of BNPP-^{14}C (up to 0.2 ml) for 30 min at 25°C. The concentration of inhibitor chosen was sufficient for at least 98% inhibition of the esterase (fig.1). The residual esterolytic activity was tested with methyl butyrate [11]. The amount of BNPP required varied considerably and was much higher than the amount necessary for total inhibition of a corresponding quantity of purified esterase. This might be due to the action of phosphodiesterases in microsomes [16]. The labeled microsomes were separated from low-molecular radioactive compounds by dialysis against 0.005 M phosphate buffer pH 8.0 (3 × 24 hr at 4°C). From measurements of the specific radioactivity it was calculated that 1 g of microsomal protein contains 1.20 ± 0.14 (standard deviation, n=6) μmol of esterase subunits. Assuming a subunit wt of 58 000, this means that 7.0 ± 0.8% (n=6) of the microsomal protein is made up of unspecific esterase. This estimation is only valid, if esterase is the only microsomal protein which binds the inhibitor.

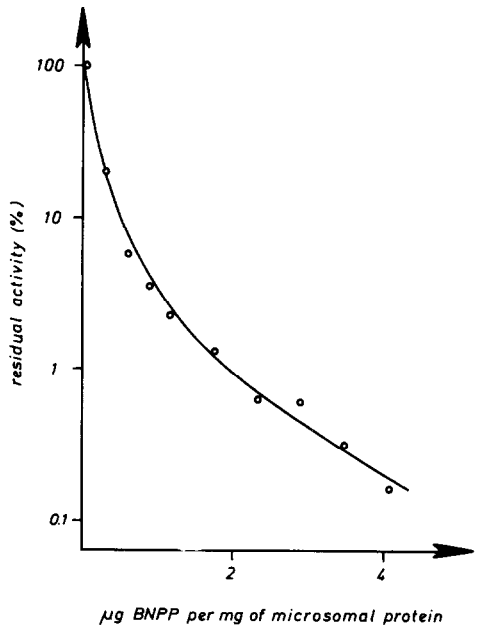

Fig. 1. Inhibition by BNPP of esterase in microsomes. Microsomes were treated with varying amounts of BNPP for 30 min at 25°C (see text). The residual esterolytic activity was tested with methyl butyrate [11].

For clarifying this point we treated labeled and dialysed microsomes with SDS and mercaptoethanol according to Weber and Osborn [17]. After SDS-discelectrophoresis (fig.2), all the radioactivity was found in one sharp band with the same mobility as purified esterase. Especially, neither some insoluble material at the top of the disc-gel, nor the low mol. wt fraction at the front showed any radioactivity.

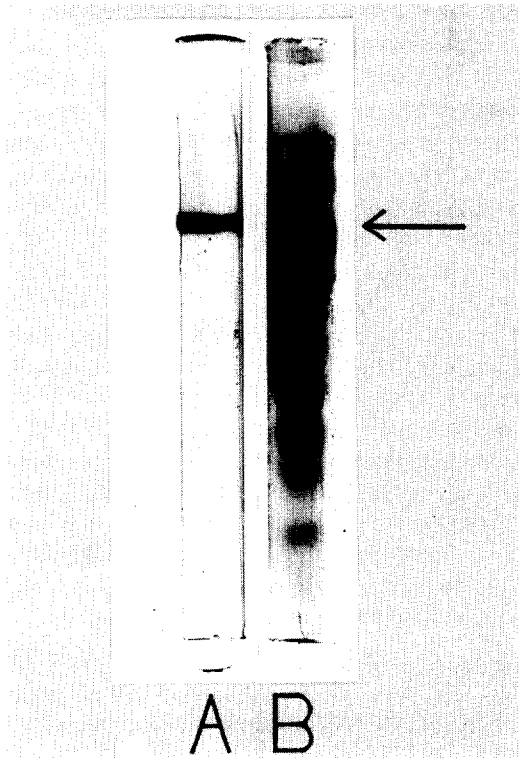

Fig. 2. SDS-disc-electrophoresis [17] of BNPP-labeled microsomes. A: Higly purified esterase run under the same conditions as B. B: BNPP-labeled microsomes; the arrow marks the sole, radioactive band. Staining with Coomassie Blue [17].

3.2. *Estimation of protein in solutions of highly purified esterase*

In order to avoid systematic errors by empirical methods of protein estimation, we used the nitrogen contents of the esterase for calculation of the protein contents. This is possible, because the theoretical value, (16.29% N), is known from amino acid analysis [18]. All factors given in table 1 are based upon this value, they were obtained by a series of estimations from a highly purified enzyme preparation. We obtained standard deviations for three of the methods listed in table 1: 3.4 % (n=6) for nitrogen determination, 0.5 % (n=10) for the biuret procedure, and 3.9 % (n=20) for the active site titration, all values measured with highly purified enzyme.

3.3. *Estimation of the equivalent weight from purification of the labeled enzyme*

At all steps of the purification procedure BNPP-^{14}C-inhibited esterase behaved as non-labeled enzyme. No re-activation occurred at any stage. The equiv. wts, obtained in several preparations, are listed in table 2. These equiv. wts represent the product of protein content per count and the specific radioactivity ($R_0/2$) of the 4-nitrophenyl phosphate residue. The equiv. wts of pig liver esterase, as determined by this procedure, are somewhat lower than values obtained by titration of the highly purified esterase with E 600. For enzyme

Table 1
Factors for the estimation of purified pig liver esterase

Method	Factor	N.B.
N-determination	0.0860	μmol of NH_3 obtained in 1 ml sample multiplied with this factor gives mg/ml
Biuret	1.91	ΔA_{546} measured at 2 cm light path in 1 ml test-solution multiplied with this factor gives mg of protein per vol of enzyme solution tested
A_{280} nm	0.719	A_{280} measured at 1 cm light path multiplied with this factor gives mg/ml; $A^{1\%}_{280} = 13.9$[a]
Titration with E600	0.058	nmol of 4-nitrophenol from initial burst multiplied with this factor gives mg of pure enzyme

[a] Barker and Jencks [9] reported $A^{1\%}_{280} = 13.8$ for pig liver esterase.

Table 2
Equivalent weights calculated from the preparation of labeled esterase

Step of preparation [11]	Equivalent weight in preparation No. 1	2	2comp.[b]	3	
DEAE-column	80 600	89 800	not det.	not det.	
2nd gel filtration	58 400	57 100	61 500[c]	60 800	
Subsequent fractionation with ammonium sulfate[a]	58 400	–	–	58 100	
Desalted fractions after isoelectric focusing	–	–	–	H-fraction[d] 58 700	N-fraction[d] 57 100
microsomes obtained by	ultra-centrifugation [11]			acid precipitation [11]	

[a] last step of the standard procedure [11]
[b] preparation from non-labeled microsomes, see text.
[c] obtained by titration with E600, ± 1100 (standard deviation of the mean, n=4).
[d] fractions with high (H) resp. low (N) isoelectric points [19,20].

prepared by one of our new procedures [11], we normally get equiv. wts between 60 000 and 70 000. The lowest value we have ever got was 60 300 ± 1 000 (standard deviation of the mean, n=4). The preparation '2 comp.' (table 2) of native enzyme was prepared under the same conditions as the radioactive preparation No.2, which yielded a lower equiv. wt.

4. Discussion

In one of our preceeding papers [1] on the subunit wt of esterases, we discussed the possibility that one of the subunits of trimeric esterases might bear no active site since, at that time, subunit wt and equivalent wt of the enzyme appeared to be markedly different. The results presented here clearly show that the equiv. wts of pig liver esterase given in earlier communications [7–9] have been too high. Here we report good agreement between the equiv. wt and the subunit wt. This might be also true for similar esterases from ox liver and pig kidney [1].

The question arises, whether the earlier preparations of pig liver esterase contained contamination by foreign proteins, or by partly denatured enzyme. Experiments No.2, and 2 comp. (table 2) indicate that partial denaturation might be a factor in lowering the titratable equiv. wt. It is reasonable that this denaturation is reduced considerably, if the enzyme is prepared in a much shorter time, according to our new methods [11]. But we cannot exclude the possibility that earlier preparations also contained some further impurities. Errors in protein estimation do not account for the higher values of the equiv. wt, because the factors reported in table 1 are essentially the same as used formerly [7].

The value of 58 000 reported here is dependent on the theoretical value of the nitrogen content of the enzyme. The latter value has an experimental error of about 5% ([18], and K. Borner, personal communication).

Compared with this, the errors of other parameters (e.g. measurement of radioactivity) are small. Therefore, we conclude that the equiv. wt of pig liver esterase is 58 000 ± 3 000.

It should be noted, however, that the relative distance between the equiv. wts of H-form and N-form [19,20] of the esterase (table 2) might be significant. This point shall be discussed in a forthcoming paper concerning the reasons of the heterogeneity of pig liver esterase.

In a study on the structure of the endoplasmic reticulum Schulze and Staudinger [21] indirectly estimated the esterase content of microsomes to be

1.4%. However, our results indicate that about 7% of the microsomal protein from pig liver cells consist of unspecific carboxylesterase. Probably the livers of other warm-blooded vertebrates contain similar amounts of the enzyme. Although the esterase from a quantitative point of view is a major protein of the microsomes, it is interesting to note that the physiological role of this enzyme is not yet known.

Acknowledgement

The skillful technical assistance of Mrs G. Marcussen-Wulff and Miss H. Rix is gratefully acknowledged. We wish to thank Professor K. Krisch and Dr J. Südi for reading the manuscript. This investigation was supported by the Deutsche Forschungsgemeinschaft.

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