RESOLUTION OF MULTIPLE FORMS OF PHENOBARBITAL-INDUCED LIVER MICROSOMAL CYTOCHROME *P*-450 BY ELECTROFOCUSING ON GRANULATED GELS

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1. Introduction

Although electrofocusing is an efficient method for separation of soluble proteins, it has not yet been of extensive use in the field of membrane biochemistry. The reason for this is mainly the hydrophobicity of membrane proteins which results in precipitation of the proteins at their isoelectric points. In order to perform isoelectric focusing of membrane proteins, we have tried to find detergents giving protein–detergent complexes suitable for electrofocusing. These complexes should not aggregate during focusing and should have constant isoelectric points.

Triton X-100 is a non-ionic detergent that seems to meet these requirements. It forms a specific homogeneous complex with the monomer of liver microsomal cytochrome *P*-450 in concentrations above 0.2% [1,2]. The complex consists of about 74 molecules of detergent per molecule of *P*-450, has a partial specific volume of 0.825 and a molecular weight of 98 000 and does not aggregate [1,2].

The present paper describes isoelectric focusing of Triton X-100–*P*-450 complexes. By this method, phenobarbital induced rabbit liver microsomal cytochrome *P*-450, called *P*-450LM$_2$ [3], is resolved into four heme-containing forms focusing between pH 7 and 9. We propose that electrofocusing may be used to resolve different types of rabbit liver microsomal *P*-450 with very similar physical characteristics, including identical molecular weights, and that homogeneity as determined by SDS–polyacrylamide gel electrophoresis is not a sufficient criterion for homogeneity of cytochrome *P*-450.

2. Materials and methods

Rabbit liver microsomal cytochrome *P*-450 with a specific content of 10–15 nmol/mg protein was prepared according to the method of Coon and collaborators [3]. The rabbits were injected intraperitoneally with phenobarbital (70 mg/kg) for five days. Liver microsomes were prepared by differential centrifugation. The microsomes were solubilized with sodium cholate and fractionated with polyethylene glycol 6000 [3]. The fraction precipitating at 10–13% (w/v) polyethylene glycol was submitted to DEAE-cellulose chromatography in the presence of 1% (v/v) Renex 690. The void fraction from the DEAE-cellulose column was applied on a hydroxyapatite column. The column was eluted with 50, 100 and 300 mM phosphate buffer containing 0.1% (v/v) Renex 690. The 100 and 300 mM phosphate fractions were used for electrofocusing.

Ampholine^R was purchased from LKB-Produkter AB (Bromma, Sweden) and Triton X-100 from Sigma Chemical Co. (St. Louis, Mo., USA). Sephadex G-75, superfine, was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Before use, 40 g of Sephadex gel was swollen in redistilled water, the fines were removed and the gel was washed with several litres of distilled water to remove charged water-soluble contaminants. The gel was then dehydrated by washing with 2 l of absolute ethanol. The gel was dried over night and used within a week.

Electrofocusing was performed in the following way. Dehydrated Sephadex G-75, about 0.4 g, was suspended in 8.5 ml redistilled water containing 1%

(v/v) of Triton X-100. Ampholine^R of suitable pH-interval was added to 2% (v/v) concentration. The suspension was stirred and applied on a glass plate, 2–8 X 23 cm (0.4 g gel covering 1 X 23 cm). The plate was placed in a stream of air until about 10–15% of the water had evaporated (determined gravimetrically). Following this procedure, the gel was quite firm, about 2 mm thick and without any visible cracks. Two small filter paper strips were soaked in 1 M NaOH and 1 M H_2SO_4, respectively and placed at each end of the plate. The plate was prefocused at 500 V for 1 h using an LKB Multiphor Basic unit. The sample was prepared in 2–4 ml of 5 mM phosphate buffer, pH 7.4, containing 1% Triton X-100 and 2% Ampholine^R. Dehydrated Sephadex G-75, 0.1–0.3 g, was added and the suspension was stirred until homogeneous. A zone corresponding to the sample suspension in volume, was scraped off from the plate about 4 cm from the anode; the sample was applied and the plate was focused at 500 V for 20 h. A strip of filter paper, corresponding to the size of the plate, was applied on the gel. When the filter paper was saturated with buffer, it was dried in an oven and stained with 0.2% Coomassie Brilliant Blue. Destaining was performed by boiling the strip in water/ethanol/acetic acid, 87:5:7.5 (by vol.) for 5–10 min. Zones on the gel plate corresponding to stained zones on the filter paper were localized and scraped off. The gels were placed in small columns and the protein eluted with 3–4 ml of 0.1 M phosphate buffer, pH 7.4. The proteins were dialyzed against 20 mM phosphate buffer, pH 7.4, with 10^{-3} M EDTA, for 3 days.

Electrofocusing on flat beds of Sephadex has certain advantages when compared to electrofocusing in columns. The technique allows accurate and easy fractionation of the protein; the fingerprint strip, stained and destained within 10 min, is placed under the glass plate and the different zones can be scraped off with high precision. Furthermore, the protein may conveniently be applied to the gel after prefocusing, at a pH where it is stable. The major disadvantage of the method is the fact that different preparations of dehydrated Sephadex G-75 do not seem to have the same resolving capacity and often extensive tailing of the protein zones occurs. The use of freshly dehydrated Sephadex tends to minimize this problem.

$NaIO_4$-supported cytochrome *P*-450-catalyzed hydroxylation of androstenedione was assayed as described previously [4]. $NaIO_4$ directly catalyzes the formation of oxygenated *P*-450 in the absence of dioxygen and therefore $NaIO_4$-supported hydroxylations are convenient to measure in experiments where the catalytic function of *P*-450 rather than of the complete hydroxylase system is to be investigated. Cytochrome *P*-450 was determined according to Omura and Sato [5] and protein according to Lowry et al. [6].

3. Results

Electrofocusing of the 100 and 300 mM phosphate buffer fractions from the hydroxyapatite column resolved the 300 mM fraction into six different bands and the 100 mM fraction into at least five different bands as evident from fig.1. Four of the bands, Nos 2, 3, 4 and 6, were common to both fractions while

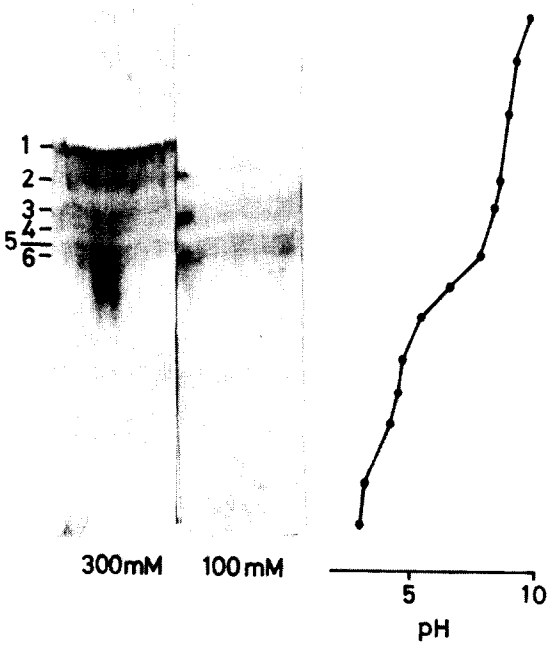

Fig.1. Electrofocusing on a flat bed of Sephadex G-75 of a purified phenobarbital induced form of rabbit liver microsomal cytochrome *P*-450 in the presence of 1% Triton X-100. The experiments were performed with 12 nmol *P*-450 from either the 300 mM (left) or the 100 mM phosphate buffer fraction obtained from the hydroxyapatite column (see text). pH was measured directly on the gel with a surface electrode.

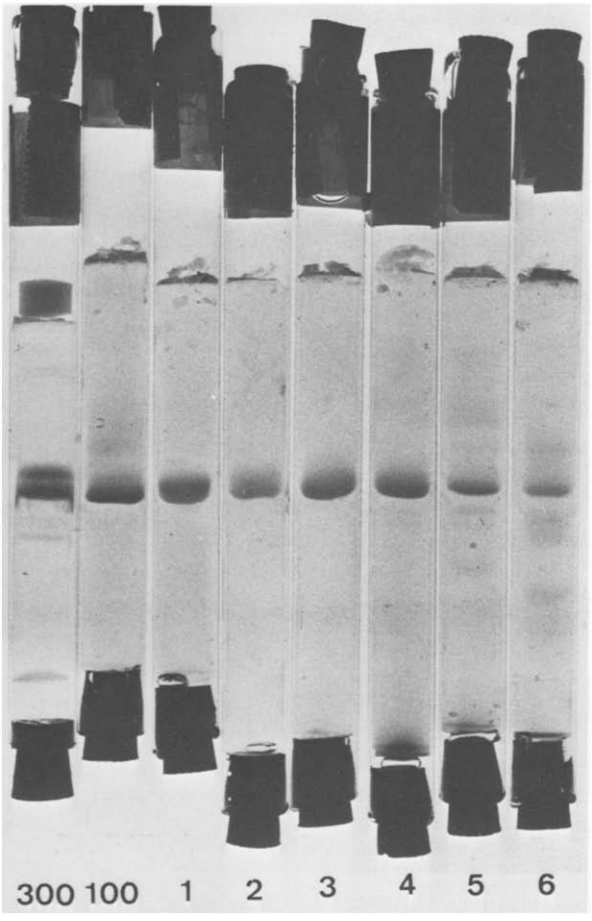

Fig.2. SDS–polyacrylamide gel electrophoresis of non-focused cytochrome *P*-450 preparations and fractions obtained after electrofocusing. Sample treatment and electrophoresis was carried out essentially as described by Weber and Osborn [7]. 20 μg of protein fractions were applied on 10% gels. Migration from top to bottom. The following samples were analyzed: 300, 300 mM phosphate buffer fraction from hydroxyapatite column; 100, 100 mM phosphate buffer fraction from hydroxyapatite column; 1–6, band Nos 1–6 from electrofocusing experiments.

band No. 1, the major zone in the 300 mM fraction, was not present in the 100 mM fraction. Furthermore, band No. 5 was only present in trace amounts in the 300 mM fraction. SDS–polyacrylamide gel electrophoresis of the 100 mM fraction and the bands obtained after electrofocusing showed that the non-focused preparation and band Nos 1–4 were essentially homogeneous (fig.2). Band Nos 5 and 6 contained some contaminating proteins that could not be detected in the non-focused material, probably due to their low concentration in this preparation.

Determination of the content of cytochrome *P*-450 in the electrofocused fractions revealed that band Nos 1, 2, 3 and 5 consisted of *P*-450, while the major constituent in band Nos 4 and 6 was cytochrome *P*-420. The catalytic specificity of the 100 and 300 mM fractions and the electrofocused fractions was investigated by incubation with androstenedione in

Table 1
6-β-Hydroxylation of androstenedione in $NaIO_4$-supported reactions catalyzed by the 300 mM and 100 mM phosphate buffer fractions from the hydroxyapatite column and by *P*-450-band Nos 1, 2, 3 and 5 obtained after electrofusing

Source	6-β-Hydroxylase activity (nmol product/nmol *P*-450)
300 mM fraction	2.70
100 mM fraction	0.36
Band No. 1 from electrofocusing[a]	5.30
Band No. 2 from electrofocusing	<0.1
Band No. 3 from electrofocusing	<0.1
Band No. 5 from electrofocusing	<0.1

[a] *P*-420-band Nos 4 and 6 did not catalyze 6-β-hydroxylation of androstenedione.

Hydroxylations were assayed in the presence of 10 mM $NaIO_4$ as described previously [4].

the presence of 10 mM $NaIO_4$ [4]. The only hydroxylase activity detected was 6-β-hydroxylation that was exclusively catalyzed by the 300 mM fraction and by band No. 1 obtained after electrofocusing (see table 1). Therefore, the absence of band No. 1 from the 100 mM fraction would seem to explain the inability of this preparation of cytochrome *P*-450 to support 6-β-hydroxylation.

4. Discussion

Liver microsomal cytochrome *P*-450 is composed of multiple forms of proteins with different physical properties [3,8]. These different forms have been characterized mainly by SDS–polyacrylamide gel electrophoresis and a tentative nomenclature has been developed on the basis of the behaviour of the different forms on the SDS–gels [3]. One cannot exclude, however, the presence of separate *P*-450 forms with identical molecular weights and it may therefore be argued that SDS–polyacrylamide gel electrophoresis is not sufficient to prove the homogeneity of a certain *P*-450 preparation. For example, Philpot and Arinç [9] have recently separated two forms of liver microsomal cytochrome *P*-450 from untreated rabbits with identical molecular weights according to SDS–polyacrylamide gel electrophoresis.

In the present paper an electrofocusing method with high resolving capacity with regard to different types of phenobarbital induced rabbit liver microsomal cytochrome *P*-450 (*P*-450LM_2 [3]) is described. A preparation of an apparently homogeneous form of *P*-450LM_2 – by SDS–gel electrophoresis criteria – has been resolved by electrofocusing in the presence of 1% Triton X-100 into four different forms, only one of which catalyzes 6-β-hydroxylation of androstenedione.

We believe that electrofocusing in the presence of 1% Triton X-100, as performed in this investigation to separate different forms of *P*-450LM_2, is an efficient method to separate membrane proteins in general. Whether the four different forms of *P*-450LM_2 obtained from electrofocusing represent four different species of *P*-450 is not clear at the present time. Work is now in progress in our laboratory to characterize the different forms with respect to substrate specificity and amino acid composition.

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