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Short Communication

Preparation of the apoenzyme and holoenzyme forms of human 17β-hydroxysteroid dehydrogenase

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ABSTRACT

The apoenzyme and holoenzyme ($NADP^+$ complex) of human placental 17β-hydroxysteroid dehydrogenase (17β-HSD) were prepared from affinity chromatography using various elutions by column liquid chromatography. The apoenzyme was obtained using NAD^+ elution in a Blue-Sepharose column, followed by NAD^+ separation on a Phenyl-Superose hydrophobic-interaction or a Mono Q anion-exchange column. The 17β-HSD–$NADP^-$ complex was prepared using $NADP^+$ elution in a Blue-Sepharose column. The two forms have different A_{280}/A_{260} ratios and are suitable for further study of enzyme–cofactor interactions.

INTRODUCTION

The key role of human placental 17β-hydroxysteroid dehydrogenase (17β-HSD, EC 1.1.1.62) in steroid conversion makes it very important to study its structure–function relationship and inhibition [1–4]. We have recently demonstrated the subunit identity of this dimeric enzyme [5] and found interactions between the subunits of 17β-HSD [6]. To study the role of coenzyme binding, it is necessary to obtain the apoenzyme. We first attempted to remove the cofactor from the enzyme using charcoal by the technique of Murdock and Koeppe [7] used for triosephosphate dehydrogenase, but 17β-HSD quickly inactivated in the presence of charcoal.

We thus modified our new rapid purification procedure for 17β-HSD based on column liquid chromatography (fast protein liquid chromatography, FPLC) [5]. As Blue-Sepharose affinity chromatography is important for the efficient homogenization of this enzyme, we used this affinity chromatographic technique and tried different cofactor elutions and further cofactor separation after this step. Fully active forms of apoenzyme and holoenzyme were obtained as described below. This method will eventually be useful for obtaining apoenzymes for other dehydrogenases.

EXPERIMENTAL

Materials

$NADP^-$, NAD^+, phenylmethylsulphonyl fluoride (PMSF), glycerol, deoxycholate and tri-

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chloroacetic acid were obtained from Sigma (St. Louis, MO, USA). 17β-Estradiol, 2-mercaptoethanol and dithiothreitol (DTT) were bought from Aldrich (Milwaukee, WI, USA). Protein markers (low molecular mass) for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), ammonium persulfate and N,N,N′,N′-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad (Richmond, CA, USA). Q-Sepharose Fast Flow and Blue-Sepharose CL-6B columns were packed in our laboratory with chromatographic media, and XK columns were from Pharmacia-LKB (Montreal, Canada). Phenyl-Superose HR 10/10 and Mono Q HR 5/5 columns were from the same company. Bicinchroninic acid (BCA) protein assay reagent was purchased from Pierce (Rockford, IL, USA). Centricon and Centri-Prep concentrators were obtained from Amicon (Beverley, MA, USA). All reagents were of the highest grade available.

Enzyme assay

17β-HSD was assayed by spectrophotometric measurement of NAD^+ reduction monitored by the absorption increase at 340 nm. The reaction mixture contained 0.5 m*M* NAD^+ and 25 μ*M* estradiol in 50 m*M* diethanolamine buffer, pH 9.1. One unit of enzyme is defined as the amount required to catalyze the formation of 1 μmol of estrone in 1 min under the above conditions (at 23° ± 1°C):

$$\text{estradiol} + NAD^+ \overset{17\beta\text{-HSD}}{\rightleftharpoons} \text{estrone} + NADH + H^+$$

Protein concentration measurements

Protein concentrations were measured using the BCA assay. Different types of interferences were eliminated by selectively precipitating the protein with deoxycholate and trichloroacetic acid [8,9].

SDS-PAGE

SDS-PAGE was carried out according to Laemmli [10] using Bio-Rad Mini-Protean II [11] and Pharmacia Phast-System. The mini gels contained 12% polyacrylamide–N′,N′-bismethyleneacrylamide (37:1). Final concentrations of SDS and glycerol in the sample were 0.5% and 10%, respectively. After migration, the gels were stained with Coomassie Brilliant Blue or immunoblotted with polyclonal anti-17β-HSD serum from rabbit and ^{125}I-labelled anti-rabbit immunoglobulin G from goat according to St-John *et al.* [12].

Purification steps

A buffer containing 40 m*M* Tris–HCl, pH 7.5, 1 m*M* EDTA, 0.2 m*M* DTT and 20% glycerol was used as the principal buffer after placenta fractionation and is hereafter referred to as buffer A. When 17β-HSD fractions were stored overnight, they were always adjusted to 50% in glycerol and kept at −20°C. All chromatographies were carried out with a Pharmacia FPLC system, which consisted of an LCC-500 controller and two P3500 pumps.

Placenta homogenization and 17β-HSD fraction. The placenta was refrigerated on ice after delivery and treated within 45 min. About 0.3 kg of tissue was obtained from one placenta ground in an Osterizer liquefier–blender, followed by centrifugation and ultracentrifugation, according to Yang *et al.* [13]. Finally, fractions precipitating between 30 and 55% saturation in ammonium sulphate were collected, diluted and loaded directly onto a Q-Sepharose column.

Chromatography. The chromatographic steps have similarities to the three-column procedure previously described [5], especially for the first Q-Sepharose anion-exchange column. After ammonium fractionation, samples of 17β-HSD were loaded on a laboratory-packed column (100 mm × 26 mm I.D.). The enzyme activity eluted at about 0.22 *M* NaCl in the same buffer. Fractions presenting at least a four-fold increase in specific activity (estimated with the aid of the A_{280} profile) as compared to the original samples were collected. These fractions were pooled, and they accounted for *ca.* fifty-fold increase in 17β-HSD specific activity from the cell extract. In this step, 0.5 m*M* PMSF was present in all buffers (in later purification steps, only 0.2 m*M* PMSF was used).

The Q-Sepharose fractions were diluted four-

fold with buffer A and loaded on a Blue-Sepharose column (70 mm × 16 mm I.D.) at a flow-rate of 3.5 ml/min. About 40% of the proteins which contained no 17β-HSD activity passed through the column. The column was then washed with 50 m*M* NaCl in buffer A, which eliminated about 10% contaminant protein.

The elution in this chromatography is specific to the preparation of the apoenzyme or of the holoenzyme as detailed in Results and discussion.

RESULTS AND DISCUSSION

Preparation of 17β-HSD apoenzyme

Blue-Sepharose chromatography. NAD^+ elution was employed in this preparation. After sample loading on the Blue-Sepharose column followed by the low-concentration NaCl wash described above, the column was re-equilibrated with buffer A. The enzyme activity was then eluted by 4 m*M* NAD^+ in buffer A at a flow-rate of 2–3 ml/min (Fig. 1). The collected fractions were about 95% homogeneous as revealed by SDS-PAGE (Fig. 2) and gel scanning (Amersham PAS system and GL-1000 software) with more than

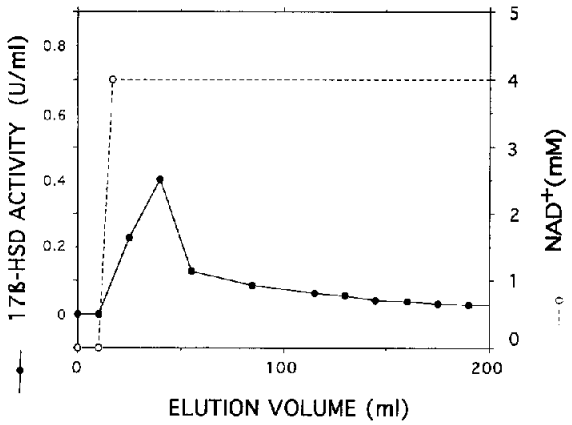

Fig. 1. NAD^+ elution of 17β-HSD from a Blue-Sepharose column to prepare the apoenzyme (dashed line). The column (70 mm × 16 mm I.D.) was saturated by a 17β-HSD sample of a total activity of 29 U. 17β-HSD activity was eluted by a one-step NAD^+ elution at 4 m*M*. The collected main fractions were more than 150 ml in volume and *ca.* 95% homogeneous. Their activity accounted for 70% yield (the protein absorbance is not shown as 4 m*M* NAD^+ gave a very high absorbance at 280 nm).

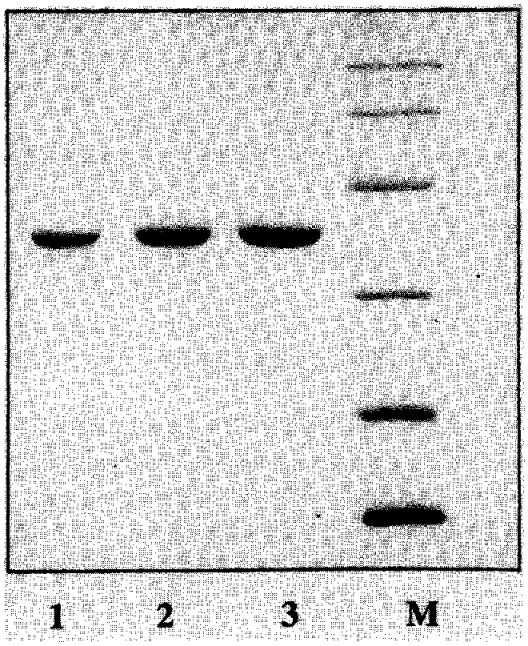

Fig. 2. SDS-PAGE of 17β-HSD from different preparations. Lanes: 1 = fractions of the apoenzyme eluted from the Mono Q column; 2 = apoenzyme fractions eluted from the Phenyl-Superose column; 3 = fractions of the holoenzyme (complexed with $NADP^+$) eluted from the Blue-Sepharose column; M = protein markers, from top to bottom; phosphorylase b (M_r 97 400), serum albumin (M_r 66 200), ovalbumin (M_r 45 000), bovine carbonic anhydrase (M_r 31 000), main soybean tyrpsin inhibitor (M_r 21 500) and lysozyme (M_r 14 400).

70% yield. To obtain a high yield with NAD^+ elution, it is critical that the column be saturated up to the maximum when loading the 17β-HSD sample. In this way, the enzyme activity was finally eluted with a high yield in a small volume.

Separation of NAD^+ from 17β-HSD. The Blue-Sepharose fractions contained a high concentration of NAD^+ which is difficult to separate by dialyses or Centricon centrifuges. These fractions were made 0.82 *M* in ammonium sulphate in buffer A and subsequently loaded on a Phenyl-Superose hydrophobic-interaction column (HR 10/10) at about 1.0 ml/min. Most NAD^+ and contaminant proteins were not retained under these conditions. The column was then washed with 0.82 *M* ammonium sulphate in buffer A, followed by a reversed gradient of 0.82–0 *M* ammonium sulphate in the same buffer. An additional

wash by holding the gradient at about 50% ammonium sulphate concentration for three or four column volumes allowed better dissociation and separation of NAD^+ from the enzyme.

Alternatively, anion-exchange chromatography proved to be as efficient as Phenyl-Superose chromatography for separating NAD^+ from the enzyme. The Blue-Sepharose fractions were loaded on a Mono Q HR 5/5 column, followed by a NaCl gradient (0–0.5 *M*, 30 ml total volume) in buffer A. 17β-HSD activity eluted at about 0.25 *M* NaCl.

Concentration of 17β-HSD preparation and analysis of its cofactor content. The main fractions from the above Phenyl-Superose chromatography are homogeneous, as shown by SDS-PAGE (Fig. 2). They were pooled, concentrated and dialysed repeatedly with buffer A using a Centricon concentrator, and finally concentrated in the same apparatus to about 1.5–2 mg/ml with a specific activity of more than 7 U/mg. After three or four dialyses, a constant A_{280}/A_{260} ratio of 1.65 ± 0.1 was obtained. By addition of estradiol, no absorption increase at 340 nm was found, indicating that no cofactor was significantly present in this preparation [14]. This is the apoenzyme of 17β-HSD, finally stored in 50% glycerol, 0.4 m*M* DTT and 40 m*M* Tris, pH 7.5 at −20°C after buffer adjustment.

The main fractions from Mono Q chromatography (4 ml per fraction) (Fig. 2) revealed an A_{280}/A_{260} ratio of about 1.7 ± 0.1, similar to that of the apoenzyme preparation using the Phenyl-Superose column. The specific activity obtained was also similar. These results indicate that the absorption of 17β-HSD on both columns facilitated the dissociation and separation of NAD^+. This will be useful for preparing other enzymes interacting with cofactors, especially labile enzymes.

Preparation of 17β-HSD holoenzyme

Using an alternative method, the Blue-Sepharose column was eluted using an $NADP^+$ gradient (0–100 μ*M*) in buffer A. 17β-HSD activity eluted at *ca.* 40 μ*M* $NADP^+$ only (Fig. 3), in a small volume where most fractions (total 20 ml) were homogeneous, as demonstrated by SDS-PAGE (Fig. 2), and the yield was as high as 80%. This indicates a very high specificity of $NADP^+$ for 17β-HSD, which makes the preparation of the holoenzyme (complexed with $NADP^+$) easier to perform than that of the apoenzyme. The high saturation of the column is not necessary for the preparation of the holoenzyme.

The above homogeneous fractions were pooled and concentrated. After three or four dialyses against buffer A in a Centricon concentrator, the A_{280}/A_{260} ratio of the preparation reached a constant value of 1.08 ± 0.05, being much lower than that of the apoenzyme. Addition of the substrate estradiol to the preparation resulted in a stoichiometric increase of absorption at 340 nm, indicating that 17β-HSD tightly complexes with one $NADP^+$ in this preparation [14]. This phenomenon shows a high affinity of $NADP^+$ for 17β-HSD, consistent with the high specificity of this cofactor demonstrated during affinity column elution. The preparation was stored similarly to the apoenzyme (see above) and the enzyme activity was well retained for many months. These two forms are being used for further study of enzyme–cofactor interactions.

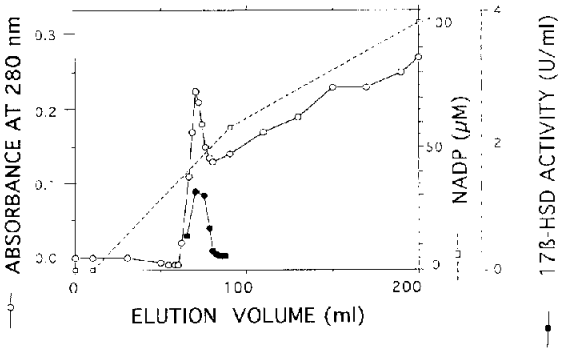

Fig. 3. $NADP^+$ elution of 17β-HSD from a Blue-Sepharose column to prepare the holoenzyme (dashed line). The column (100 mm × 16 mm I.D.) was loaded with a sample of about 8 U in activity. 17β-HSD eluted at about 40 μ*M* $NADP^+$ in the gradient. The elution volume in which most fractions (total volume 20 ml) were homogeneous was small. The yield was *ca.* 80%. This indicates a high specificity of $NADP^+$ for 17β-HSD.

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