Pergamon

0960-0760(94)00144-8

J. Steroid Biochem. Molec. Biol. Vol. 52, No. 1, pp. 77–81, 1995

0960-0760/95 $9.50 + 0.00

Human 17β-Hydroxysteroid Dehydrogenase: Optical Properties of its Complex with NADP+

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The apoenzyme of the human placental 17β-hydroxysteroid dehydrogenase (17β-HSD) and its complex with NADP+ were prepared from two alternative procedures. The apoenzyme (Form I) has an absorption maximum at about 279 nm, and an absorption ratio at 280 and 260 nm of 1.65 ± 0.1; whereas the complex (Form II) has a broad absorption peak between 268–278 nm, and a 280 to 260 nm ratio of 1.1 ± 0.05. Upon addition of the substrate estradiol to the complex, an absorption increase at 340 nm and a fluorescence emission at 450 nm, following NADPH formation, were produced. Both changes indicate that one cofactor is tightly bound to the 17β-HSD molecule in this complex. No significant optical change can be produced in this way for the apoenzyme. Convenient analyses of cofactor content of the enzyme are thus provided. The optical analyses and the homogeneous apo- or holo-enzyme preparations are important in the study of the enzyme's function and crystallization. This is the first human steroid converting enzyme which has yielded X-ray quality crystals.

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INTRODUCTION

17β-Hydroxysteroid dehydrogenase (17β-HSD)[1] [EC1.1.1.62] from the soluble subcellular fraction of human placenta is responsible for the formation of active estrogens, thus stimulating the development of breast tumors [1–3]. It is pivotal both in endocrinology and cancer therapy. It has been proposed that 17β-HSD has a dimeric structure, but different opinions were reported concerning the subunit identity [4–7]. Our recent results proved that 17β-HSD is a homodimer using a combined study of enzymology and molecular biology [3]. As 17β-HSD is efficiently purified using affinity chromatography with coenzyme elution, it is important to know the coenzyme content in different preparations [3, 8–10]. This is very useful in the further study of subunit interactions, ligand binding and crystallization of this enzyme.

In the present work we demonstrate the marked influence of the coenzyme on the absorption spectra of 17β-HSD and the influence of the enzyme on the fluorescence of reduced coenzyme. This can be used to determine different stoichiometries of coenzyme association with 17β-HSD. The apoenzyme and holoenzyme forms are important for further binding and structural studies of 17β-HSD.

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Received 8 July 1994; accepted 22 Aug. 1994.

MATERIALS AND METHODS

Materials

NAD^+, $NADP^+$, NADPH, phenylmethane sulfonyl fluoride (PMSF), glycerol and Tris-base were purchased from Sigma. Estradiol and dithiothreitol (DTT) were from Aldrich. The Q-Sepharose Fast Flow and blue-Sepharose CL-6B columns were packed by ourselves with Pharmacia-LKB chromatographic media and XK columns, while the phenyl-Superose HR10/10 column was directly from the same company. Centricon and Centri-prep concentrators for sample concentration and buffer exchange were from Amicon. All reagents were of the highest grade available.

Enzyme assay

Activity of 17β-HSD was assayed by spectrophotometric measurement of NAD^+ reduction indicated by the absorbance increase at 340 nm and 23 ± 1°C. The reaction mixture contained 0.5 mM NAD^+, 25 μM estradiol in 50 mM $NaHCO_3$–Na_2CO_3 buffer,

pH 9.2. At this pH the maximum activity of estradiol oxidation is obtained. A blank value lacking estradiol was obtained under the same condition and subtracted.

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

One unit of enzyme is defined as the amount of enzyme that catalyzes the formation of 1 μmol of product in 1 min under the above conditions.

Enzyme purification

Placental 17β-HSD was purified in two alternative ways. The first procedure involves three fast protein liquid chromatography (FPLC) chromatographies after cell extraction and ammonium sulfate fractionation: Q-Sepharose ion exchange, blue-Sepharose affinity (with NAD^+ elution) and phenyl-Superose hydrophobic interaction chromatographies [3]. Alternatively, the second procedure consists of only the first two chromatographies, in which the blue-Sepharose chromatography was carried out with $NADP^+$ elution [11]. These preparations yield homogeneous 17β-HSD as revealed by sodium dodecyl sulfate (SDS) and native gel electrophoreses. They have similar high specific activities catalyzing the formation of 7.5–8.0 μmol estrone from estradiol per min per mg enzyme protein under the above-mentioned assay conditions [3].

Protein concentration determinations

The bicinchoninic acid protein assay [12] was used to determine protein concentrations. Protein samples were precipitated with deoxycholate and trichloroacetic acid to eliminate different types of interference [13, 14]. The optical method of Warburg and Christian [15] gave a slightly lower value ($\sim$12%) than the bicinchoninic acid assay. This is similar to the results of Jarabak and Street [4] where they found that this optical method gave protein concentration values of about 10% less than the values deduced from the weights of the constituent amino acids (after correction for water of hydrolysis).

Absorption measurements

Absorption spectra were measured with a Beckman DU-70 Spectrophotometer. When necessary, a microcuvette of 50 μl was used. Spectra were stored each time after scanning. A molar extinction coefficient of 6220 is used for the reduced form of $NADP^+$ at 340 nm. A buffer containing 40 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.2 mM DTT, and 20% glycerol was used as the principal buffer and is referred to herein as buffer A. The background contributed by buffer is subtracted for all spectra.

Fluorescence measurement

Fluorescence spectra were obtained using an SLM-8000 fluorometer. NADPH fluorescence emission was measured at 450 nm following excitation at 350 nm. The slit width for excitation was 8 nm while that for emission was 16 nm.

RESULTS

Different preparations of 17β-HSD leading to different A_{280}/A_{260} ratio

Using the three step chromatography procedure (see Methods), we were able to obtain a preparation with an A_{280}/A_{260} absorption ratio of 1.65 ± 0.1. This preparation, collected from the phenyl-Superose fractions, is referred to as form I which will be identified as the apoenzyme below. The A_{280}/A_{260} of form I remained constant after 3–4 times centricon centrifuge to "dialyse" the free cofactor. Alternatively, when blue-Sepharose affinity chromatography and $NADP^+$ elution are used in the last step, the A_{280}/A_{260} ratio is 1.1 ± 0.05, and repeated centricon centrifuge cannot increase this ratio. This preparation is referred to as form II that will be identified as the holoenzyme below. Both preparations have the same specific activity, catalyzing the formation of 7.5–8.0 μmol estrone from estradiol per min per mg enzyme protein under the aforementioned assay conditions.

Absorption spectra of 17β-HSD apoenzyme and its complex with $NADP^+$

Form I exhibited a spectrum with a maximum at 278–279 nm [Fig. 1(A, a); Table 1], while form II exhibited a relatively broad peak between 268 and 278 nm which may be constituted by two small maxima at 268 and 278 nm [Fig. 1(A, d)]. Upon addition of estradiol (20 μM final concentration) to Form II (4.57 μM final concentration), it exhibited an important absorption increase at 340 nm due to $NADP^+$ reduction. It is equivalent to 4 μM NADPH formation or a stoichiometry of 0.87 ± 0.05 of the enzyme concentration [Fig. 1(B, d)] as the constant A_{280}/A_{260} value indicated almost all $NADP^+$ are tightly bound under the experimental conditions. While under the same conditions, form I exhibited a minute absorption increase at 340 nm upon estradiol addition [corresponding to about 0.1 stoichiometry of the enzyme concentration, Fig. 1(B, b)]. This lies within the limit of experimental errors.

When an equal concentration of $NADP^+$ was added to form I, the A_{280}/A_{260} ratio shifted to 1.1 ± 0.05, while the absorption spectra demonstrated a broad peak region between 268–278 nm [Fig. 1(A, b)]. Again, this ratio is constant during repeated centricon centrifuge. This was very similar to the behavior of form II. Moreover, upon addition of a saturating estradiol concentration as above, a 340 nm absorption increase indicated 0.7 ± 0.05 stoichiometry of enzyme concentration in $NADP^+$ reduction, the same as in the case of form II (data not shown). When a 2-fold concentration of $NADP^+$ was added to form I solution, the absorption maximum shifted to 268 nm and the ratio A_{280}/A_{260}

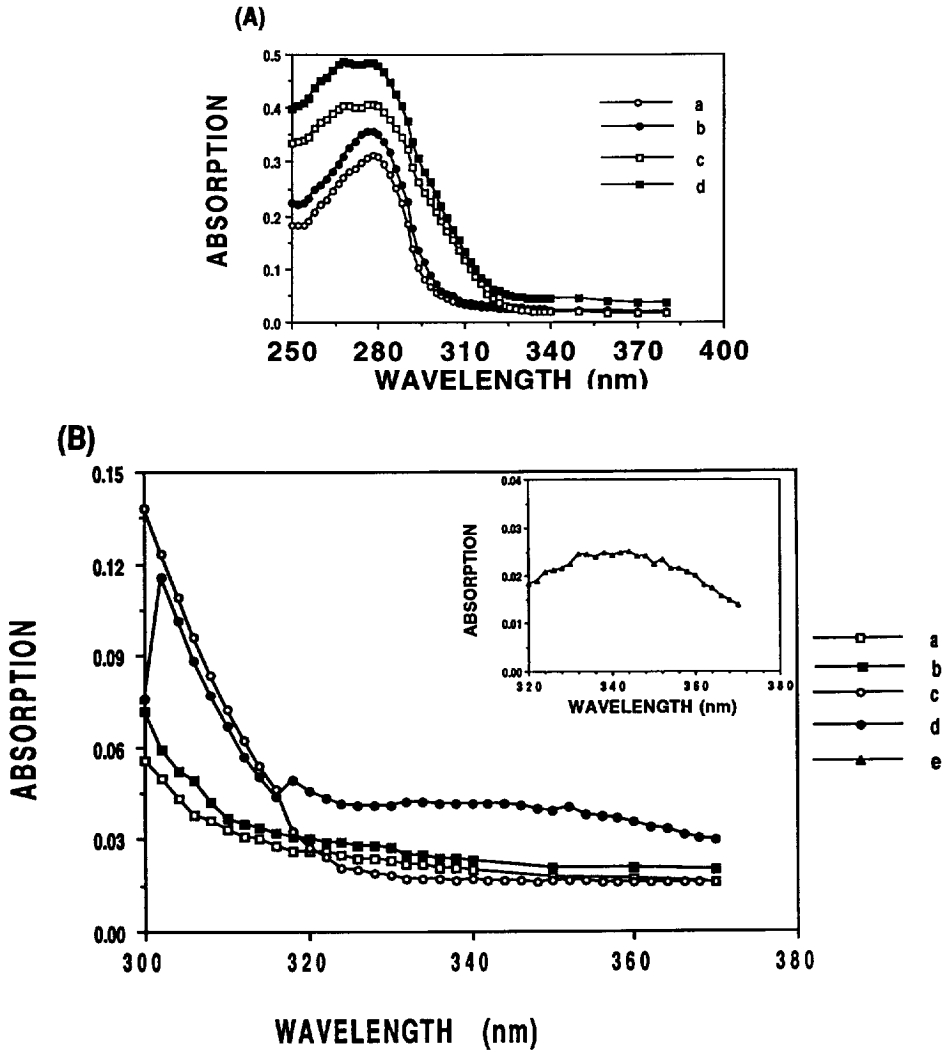

Fig. 1. Absorption spectra of 17β-HSD. (A) UV absorption spectra: (a) The apoenzyme (4.57 μM) in buffer A; (b) same as (a), plus 4.57 μM NADP⁺; (c) same as (a), plus 9.14 μM NADP⁺; (d) the holoenzyme (5 μM) in buffer A, prepared as mentioned in the text, with a peak between 268 and 278 nm. (B) Spectra near NADPH absorption region: (a) The apoenzyme (4.57 μM) in buffer A; (b) saturating estradiol (20 μM final) was added to the sample in (a); (c) the holoenzyme (4.57 μM) in buffer A, prepared as mentioned in the text; (d) saturating estradiol (20 μM final) was added to the sample in (c). The created peak at 340 nm denotes that NADP⁺ reduction being apparently 4 μM calculated from free NADP⁺ absorption coefficient; (e) the insert is the difference between (d) and (c), indicating the contribution of NADPH⁺ formation with a maximum at 340 nm.

became 0.88 ± 0.05 [Fig. 1(A, c)]. This value cannot be used to estimate the true stoichiometry, as it is not constant in the course of repeated centricon centrifugations. It is however a useful value to verify the existence of extra-NADP of form I. This is also why the values reported in Table I are very useful to indicate different NADP⁺ content of a 17β-HSD sample. The above results demonstrated that whereas form I was free of coenzyme, the form II 17β-HSD molecule bound strongly one NADP⁺ molecule. Therefore the forms I and II are, respectively, the apoenzyme and holoenzyme. The somewhat hypochromic effect is similar to the results reported by Velick and Furfine [16] and Murdock and Koeppe [17] which are further studied as follows.

Table 1. Absorption characteristics of 17β-HSD complexes with NADP⁺

Characteristics	Numbers of NADP⁺ added per apoenzyme molecule			Holoenzyme prepared (see methods)
	0	1	2	
A_{280}/A_{260}	1.65 ± 0.1	1.1 ± 0.05	0.88 ± 0.05	1.1 ± 0.05
Absorption maxima (nm)	278–279	268–278	268	268–278

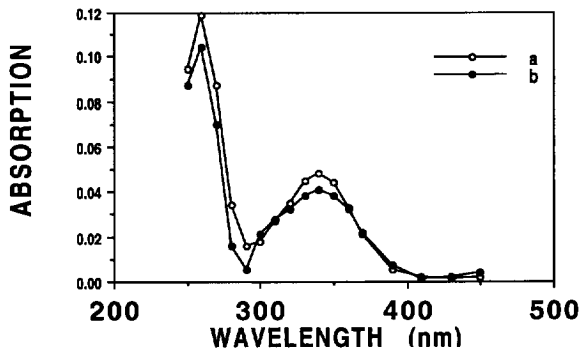

Fig. 2. The hypochromic effect of NADPH absorption in the presence of 17β-HSD. (a) NADPH (8 μM) in Buffer A in the absence of 17β-HSD. (b) NADPH (8 μM) in the presence of 17β-HSD apoenzyme (4 μM) (the apoenzyme contribution is subtracted).

A comparison of NADPH absorption in the absence and in the presence of 17β-HSD was carried out. The absorbance contribution of NADPH at 340 nm in the presence of 4 μM 17β-HSD ([NADPH]/[17β-HSD] = 2) is obtained after subtracting the enzyme contribution [Fig. 2(b)]. We can see clearly that the absorption contributions of NADPH in the presence or absence of 17β-HSD are comparable. A hypochromic effect, which accounts for about 19% of free NADPH absorption, however, is shown in repeated experiments when the enzyme is present. This is similar to many reports in the literature [e.g. 13, 14]. So, the 0.87 stoichiometry of $NADP^+$ in the complex reflects a corrected value close to 1.

Fluorescence emission of NADPH following an excitation at 350 nm

The apoenzyme of 17β-HSD has no fluorescence emission at 450 nm following an excitation at 350 nm.

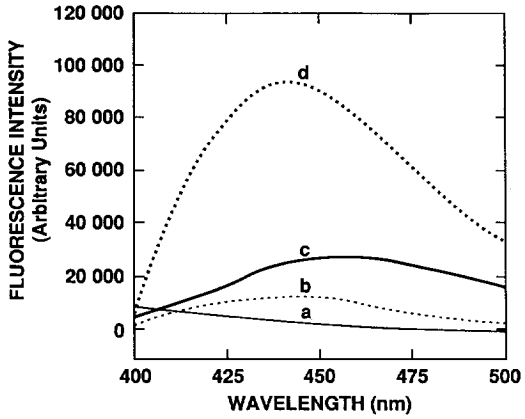

Fig. 3. Fluorescence spectra of 17β-HSD. (a) The apoenzyme (1 μM) in buffer A which does not fluoresce at 450 nm. (b) The addition of 1 μM $NADP^+$ to the apoenzyme. (c) Free NADPH (1 μM) in the same buffer. (d) Form II complex solution (1 μM), saturated with estradiol (the contribution of 17β-HSD and estradiol are subtracted). Spectra (c) and (d) indicate that the presence of 17β-HSD enhances NADPH fluorescence and shifts the maximum from about 460 to 440 nm.

Nevertheless, the complex with $NADP^+$ exhibited a slight emission at 450 nm [Fig. 3(b)]. Upon addition of estradiol (final concentration 20 μM) to an apoenzyme solution of a relatively high concentration (3 μM), the resulting fluorescence increase was minute and within the limit of experimental errors (data not shown). The addition of the same concentration of estradiol to the form II complex solution (1 μM) resulted in an important fluorescence increase, due to NADPH formation [Fig. 3(d)]. These results are in good agreement with the above-mentioned experiments in absorption. When comparing Fig. 3(d) with Fig. 3(c), we can see that the presence of 17β-HSD enhances NADPH fluorescence and shifts the maximum from about 460 to 440 nm. This is also verified by direct addition of 17β-HSD to free NADH solution (data not shown).

DISCUSSION

Using the above mentioned optical methods, we identified homogeneous apo- and holoenzyme forms. With these preparations, further study on enzyme–ligand interactions are being carried out and 17β-HSD is successfully crystallized [18]. This is the first report on crystals suitable for complete X-ray structure determination of a human steroid-converting enzyme. The optical method is simple, clear, and useful for the study of other dehydrogenases. The specific activity of 17β-HSD reported here (7.5–8.0 U/mg) is slightly higher than before (e.g. 7.2 U/mg in [3]). That may be due to a maxima acceleration and simplification of the purification, which can further eliminate the microheterogeneity of 17β-HSD [3, 10]. Such higher specific activity has been obtained in repeated enzyme preparations.

The minimal affinity of $NADP^+$ to 17β-HSD can be estimated as follows: as mentioned in Results, the A_{280}/A_{260} ratio remained constant for form II after several "dialyses", i.e. the free $NADP^+$ concentration should be much smaller than the bound $NADP^+$ concentration, $[NADP^+] \ll [E \cdot NADP^+]$ (or $[E \cdot NADP^+]/[NADP^+] > 10$). As when $NADP^+$ is added to an equal concentration of form I, the A_{280}/A_{260} ratio and the absorption spectra are identical to those of form II, the molecular composition should be the same in the two cases. As the total enzyme concentration is equal to the total $NADP^+$ concentration, the enzyme molecules should also exist mostly in the bound state, i.e. free $[E] \ll [E \cdot NADP^+]$. The minimal affinity can be estimated:

$$\text{ka} = \frac{[E \cdot NADP^+]}{[NADP^+][E]} > \frac{10}{[E]} > \frac{10}{\frac{1.5\ \mu M}{10}} = 6.7 \times 10^7\ M$$

as $[E] < 1/10[E \cdot NADP^+] < 1/10[E]_0$, while the lowest $[E]_0$ (total concentration of 17β-HSD) in centricon was about 1.5 μM.

That is to say, 17β-HSD has a strong binding site to NADP$^+$, with a $K_D < 0.015\,\mu M$. This is coincident with the strong elution property of NADP$^+$ in the enzyme purification [11]. The present results not only helped in the purification of different enzyme forms, but also helped in 17β-HSD crystallization. In fact, due to the strong binding of NADP$^+$ and the need of enzyme–coenzyme interaction study, 17β-HSD–NADP$^+$ complex was recently crystallized [10]. This is the first report on the successful crystallization of any steroid-converting enzyme from a human source.

Acknowledgements—We thank Dr F. Labrie for his interest in this work. We also thank Dr J.-Y. Wang for his help in computer programming. We thank Dr J. Lapointe for his careful reading of the manuscript. This work is supported by the Medical Research Council of Canada and a Scholarship award to S.-X.L. from "Le Fonds de la Recherche en Santé du Québec".

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