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MODIFICATION OF THE KINETIC PROPERTIES OF 5-ENE,3β-HYDROXYSTEROID DEHYDROGENASE OF HUMAN PLACENTAL MICROSOMES BY HYDROGEN PEROXIDE AND 2-MERCAPTOETHANOL

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SUMMARY

The kinetic properties of 5-ene,3β-hydroxysteroid dehydrogenase of human placental microsomes were modified by H_2O_2 and 2-mercaptoethanol. With microsomes prepared in the presence of 2-mercaptoethanol the K_M values were 0.035 μM for pregnenolone and 1.9 μM for NAD^+. When the microsomes were treated with H_2O_2 the values were 1.20 μM for pregnenolone and 30.0 μM for NAD^+. The H_2O_2 effect was reversed by 2-mercaptoethanol. Identical effects were seen with detergent-solubilized enzyme.

With H_2O_2-treated enzyme progesterone, androstenedione and 20α-hydroxyprogesterone were noncompetitive inhibitors with K_I values of 5.5, 0.83 and 4.1 μM respectively.

The results indicate that the enzyme can exist in at least two kinetically different forms and that the K_M values for NAD^+ and pregnenolone *in vivo* may be significantly lower than previously thought.

INTRODUCTION

In mammalian steroid producing tissues the conversion of pregnenolone to progesterone is catalysed by two membrane-associated enzymes: a 5-ene,3β-hydroxysteroid dehydrogenase (E.C.1.1.1.51) and a 5-ene-,3-ketosteroid isomerase (E.C.5.3.3.1). Because the human placenta is a major source of progesterone during pregnancy the kinetic properties and specificity of 3β-HSD from this tissue have been the subjects of a number of studies[1–6].

Wiener and Allen reported that 3β-HSD was inhibited by 20α-hydroxyprogesterone, a steroid present in the placenta, and proposed that this latter compound might influence the rate of progesterone formation *in vivo*[1]. In later experiments the inhibitory effects of other endogenous C_{18}, C_{19} and C_{21} steroids were demonstrated[2]. In those studies an isotopic method based on measurements of progesterone formation from 4-[^{14}C]-pregnenolone was used to determine initial velocities. Such a method has the disadvantage that neither the specific oxidation of the 3β-hydroxyl group nor the isomerization of the 4-ene bond is observed. As a result it is not clear in inhibition studies if only a single reaction or both the dehydrogenation and isomerization reactions are being affected.

Townsley reported that pregnenolone competitively inhibited dehydroepiandrosterone oxidation by 3β-HSD[3]. He did not examine 3β-HSD with pregnenolone as substrate thus the inhibitions by steroids cannot be readily interpreted in terms of progesterone formation. Ferre *et al.*[4] used a spectrophotometric assay procedure to examine the inhibitory effects of various steroids. By measuring NADH formation directly they demonstrated that the dehydrogenation step was inhibited. However, in this study also, the types of inhibition and K_I values for the various steroids were determined with dehydroepiandrosterone as substrate and not pregnenolone, thus the significance of the data for progesterone formation is unclear.

Based on these observations we felt that the kinetic properties of 3β-HSD warranted further study; in particular with pregnenolone as a substrate and with an assay procedure specific for the dehydrogenation reaction.

During the course of testing the effects of various oxidizing and reducing agents, we noted that exposure of microsomes to hydrogen peroxide or 2-mercaptoethanol significantly modified the kinetic behavior of the enzyme and these effects had implications for the *in vivo* control of progesterone formation. In this report the effects of H_2O_2 and 2-mercaptoethanol are shown and the inhibitory effects of various steroids are considered with regard to the NAD^+-linked oxidation of the 3β-hydroxyl group.

METHODS AND MATERIALS

Abbreviations used: 3β-HSD, 5-ene,3β-hydroxysteroid dehydrogenase; Bicine, *N,N-bis* (2-hydroxy-

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ethyl) glycine; Triton X-100, polyoxyethylene octylphenol; pregnenolone, 3β-hydroxy-5-pregnen-20-one; androstenedione, 4-androstene-3,20-dione; dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; 20α-hydroxyprogesterone, 20α-hydroxy-4-pregnen-3-one.

Preparation of microsomes. All procedures were carried out at 4°C. Two 30 g portions of fresh placental tissue were each homogenized in 30 ml of buffer containing 0.25 M sucrose, 10 mM 2-mercaptoethanol and 0.1 M Bicine, pH 9.0 in a Waring Blender (3 × 10 s). The homogenates were combined, centrifuged at 10,000 *g* for 10 min and the pellet discarded. The supernate was then spun at 105,000 *g* for 60 min. The pellets were suspended in 48 ml of buffer, the centrifugation at 105,000 *g* repeated and the resultant pellets suspended finally in 4.0 ml of buffer. 2-Mercaptoethanol was routinely used because it significantly enhanced the stability of 3β-HSD.

Protein determination. The fluorescamine procedure of Böhlen *et al.*[7] was used to determine protein. Fluorescence was measured at 475 nm (390 nm excitation) with ovalbumin as the protein standard.

Phospholipid determination. The procedure for phospholipid extraction and determination has been described[8].

Enzyme activity measurements. 3β-HSD activity was measured fluorometrically in an Aminco-Bowman spectrophotofluorometer fitted with a constant temperature cuvet chamber maintained at 25°C. Reaction mixtures of 1.0 ml total final volume contained 0.17 M Bicine, pH 9.0, microsomal protein, NAD^+ and substrate. The concentrations are given in the data for particular experiments. Reaction was started by the addition of steroid substrate in methanol. Stock dilutions were prepared such that the methanol concentration in any experiment was constant and did not exceed 4% (v/v). NADH fluorescence at 450 nm (340 nm excitation) was recorded continuously and the initial velocity determined from the slope of the initial linear portion of the progress curves. Protein concentrations were adjusted such that the rate of NADH formation was linear for 3–5 min. Appropriate standards were used to convert fluorescence changes (ΔF/min) to units of concentration.

Analysis of kinetic data. Values for K_M and V_{max} were estimated graphically from plots of v versus $v/[S]$[9]. Inhibition patterns and K_I values were determined from plots of v^{-1} versus $[I]$ and $[S]/v$ versus [I][10, 11].

Density gradient centrifugation. For density gradient centrifugation 0.5 ml of sample was layered onto 11.5 ml of a 10–50% (w/v) sucrose gradient in tubes calibrated at 0.5 ml intervals. After centrifugation at 37,500 rev./min for 16–17 h (4°C, Beckman L3-50, SW41 rotor) 0.5 ml fractions (24 total) were taken by use of a capillary pipet.

Materials. Reagents were from commercial sources as follows: steroids (Steraloids), Bicine (Calbiochem), NAD^+ (P-L Biochemicals), Triton X-100 (Sigma).

RESULTS

Reaction progression curves and initial velocity estimates

The effects of protein levels on reaction progression curves are illustrated in Fig. 1. At a protein concentration of 28 μg/ml the rate of NADH formation was linear for at least four min. As the levels of protein were increased the time during which the progression curve was linear decreased significantly. No formation or destruction of NADH was observed in the absence of steroid and background changes in absorbance due to organic solvent effects on the light-scattering properties of the microsomal vesicles[12] did not interfere with the fluorescence measurements.

Effects of H_2O_2 and 2-mercaptoethanol on V_{max} and K_M for pregnenolone and NAD^+

In preliminary experiments we noted that NADH formation with time was linear with H_2O_2-treated enzyme at protein concentrations at which curvilinear plots were observed with enzyme exposed to 2-mercaptoethanol. To clarify the basis for this effect V_{max} and K_M values for pregnenolone and NAD^+ were determined at pH 9.0. With microsomes prepared in buffered sucrose containing 2-mercaptoethanol at 10 mM the K_M for pregnenolone was 0.033 μM (Fig. 2). After exposure to H_2O_2 the value was increased to 1.20 μM. A subsequent exposure to 2-mercaptoethanol in excess of the added H_2O_2 resulted in a lowering of the K_M to 0.032 μM, demonstrating the reversibility of the effect. With three separate enzyme preparations the mean values ± S.D. were 0.035 ± 0.013 μM ($n = 7$) for 2-mercaptoethanol-treated

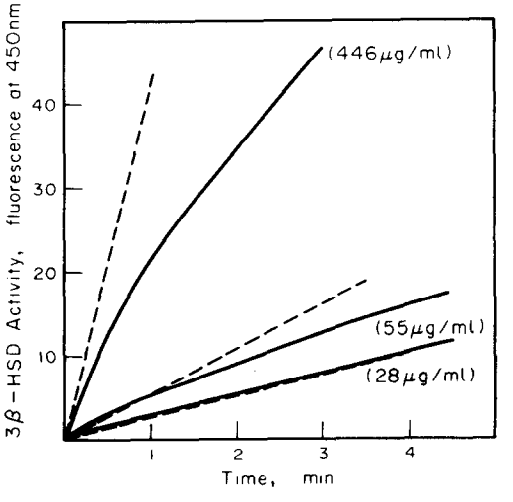

Fig. 1. 3β-HSD reaction progression curves as a function of protein concentration. The solid lines are tracings of the actual recordings of the increase of fluorescence at 450 nm as a function of time. Protein concentrations are indicated in parentheses. The dashed lines are initial velocity estimates based on the data at the lowest protein concentration (28 μg/ml). Reaction mixtures of 1.0 ml contained 0.17 M Bicine, pH 9.0, 1.0 mM NAD^+, 10 μM pregnenolone, 2% (v/v) methanol and protein as indicated. Fluorescence is given in arbitrary units. NADH at 0.5 μM gave a fluorescence of 57 units.

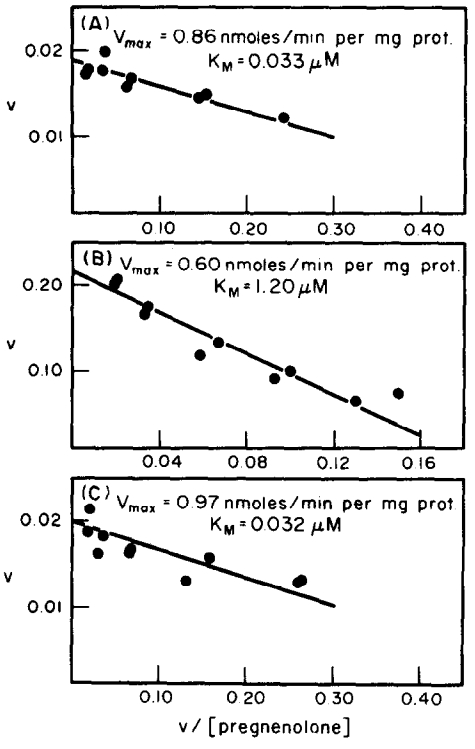

Fig. 2. Effects of H_2O_2 and 2-mercaptoethanol on V_{max} and the K_M for pregnenolone with 3β-HSD. (A) A sample of microsomal suspension in 0.25 M sucrose, 0.10 M Bicine, pH 9.0, 10 mM 2-mercaptoethanol containing 44.6 mg of protein per ml was diluted 1:20 and 0.01 ml aliquots assayed in 1.0 ml reaction mixtures containing 0.17 M Bicine, pH 9.0, 1.0 mM NAD^+ and 0.05 to 1.0 μM pregnenolone. (B) A 0.5 ml aliquot of microsomes containing 22.3 mg of protein in 0.25 M sucrose, 0.1 M Bicine, pH 9.0, 10 mM 2-mercaptoethanol was combined with 0.1 ml of 120 mM H_2O_2 and incubated at 0°C for 1 h. Then 0.01 ml aliquots were taken for assay in 1.0 ml reaction mixtures as described in (A) above. (C) To a 0.2 ml sample of oxidized microsomes from (B) 0.04 ml of 0.1 M 2-mercaptoethanol was added. After being incubated at 0°C for 1 h the sample was diluted 1:15 with 0.25 M sucrose containing 0.1 M Bicine, pH 9.0, and 10 mM 2-mercaptoethanol and 0.01 ml aliquots taken for assay as in part (A). Units are v = nmol/ml/min and $v/[\text{pregnenolone}]$ = nmol/ml/min/μM.

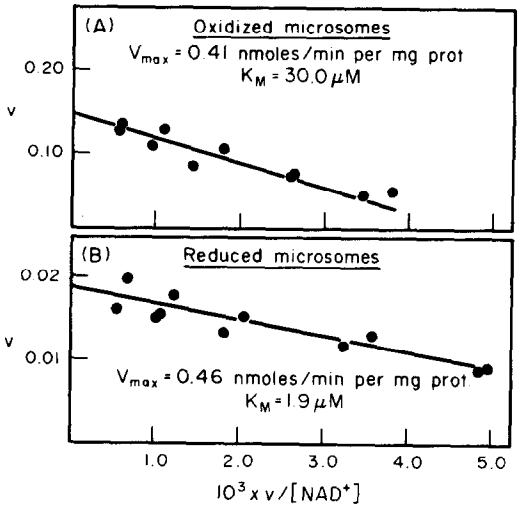

Fig. 3. Effects of H_2O_2 and 2-mercaptoethanol on V_{max} and the K_M for NAD^+ with 3β-HSD. (A) A 0.5 ml sample of microsomes in 0.25 M sucrose, 0.10 M Bicine, pH 9.0, containing 22.3 mg of protein and 20 mM H_2O_2 was incubated at 0°C for 30 min. Then 0.01 ml aliquots were taken and assayed in reaction mixtures of 1.0 ml final volume containing 0.17 M Bicine, pH 9.0, 10 μM pregnenolone and 15 to 240 μM NAD^+. (B) A 0.2 ml sample of H_2O_2-treated microsomes was made 15 mM in 2-mercaptoethanol and incubated at 0°C for 60 min. The sample was then diluted 1:15 with buffer containing 0.25 M sucrose, 0.1 M Bicine, pH 9.0, and 10 mM 2-mercaptoethanol. Following this, 0.01 ml aliquots were assayed in 1.0 ml reaction mixtures containing 0.17 M Bicine, pH 9.0, 10 μM pregnenolone and 1.9 to 30 μM NAD^+. In the figure the units are v = nmol/ml/min and $v/[NAD^+]$ = nmol/ml/min/μM.

enzyme and 1.08 ± 0.089 μM ($n = 4$) for H_2O_2-treated enzyme ($P < 0.01$). The values for V_{max} were not significantly changed. The K_M for NAD^+ was also increased after exposure of microsomes to H_2O_2 as illustrated in Fig. 3. With H_2O_2-treated microsomes the K_M for NAD^+ was 30.0 μM. After exposure to 2-mercaptoethanol the K_M was 1.9 μM. In a second experiment with a separate enzyme preparation the K_M values were 37.3 μM (H_2O_2-treated) and 5.1 μM (2-mercaptoethanol-treated).

Effects of H_2O_2 and 2-mercaptoethanol on detergent-solubilized 3β-HSD.

Because 3β-HSD is membrane bound the possibility exists that the kinetic effects of H_2O_2 and 2-mercaptoethanol reflect modifications of membrane components other than 3β-HSD. To consider this question 2-mercaptoethanol-treated microsomes were solubilized with the non-ionic detergent Triton X-100 and the effects of H_2O_2 on V_{max} and the K_M for pregnenolone were examined (Fig. 4). Solubilization resulted in a 30–60% reduction in V_{max} (compare Fig. 2A and Fig. 4A) however the K_M values for pregnenolone were not significantly different from those for the membrane-bound enzyme and the H_2O_2 effect was reversible.

A subsequent centrifugation of the solubilized microsomes yielded the distribution pattern shown in Fig. 5. The peak of 3β-HSD activity occurred near the top of the gradient and was displaced from the peaks of protein and phospholipid.

Inhibition of 3β-HSD by steroids

The possible roles for endogenous steroids in controlling 3β-HSD activity *in vivo* have been based on estimates of K_I and comparisons of these values with the levels of steroid in the placenta[1–3]. In view of the marked effect of 2-mercaptoethanol in lowering the K_M for pregnenolone, the K_I values and the inhibition patterns for progesterone, androstenedione and 20α-hydroxyprogesterone were re-examined. The effects of these compounds were tested on both 2-mercaptoethanol- and H_2O_2-treated 3β-HSD.

Because of the low K_M value for substrate with the 2-mercaptoethanol-treated microsomes, we could make only semi-quantitative estimates of K_I with the

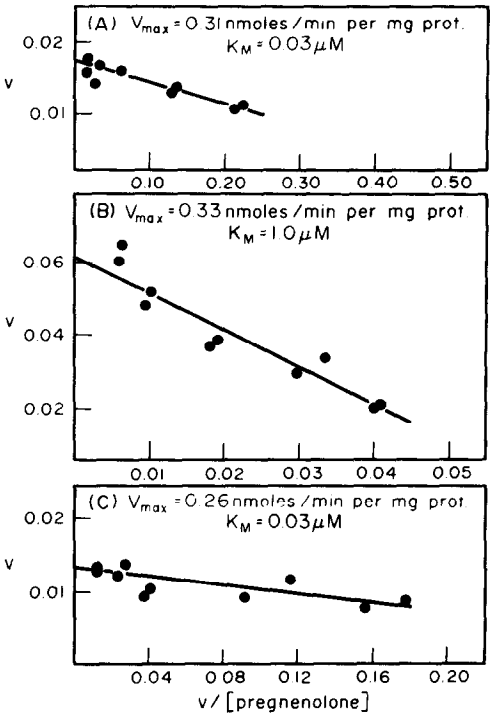

Fig. 4. Effects of H_2O_2 and 2-mercaptoethanol on V_{max} and the K_M for pregnenolone with detergent solubilized 3β-HSD. (A) A 2.0 ml sample of freshly prepared microsomes containing 5.1 mg of protein in buffer consisting of 0.25 M sucrose, 0.10 M Bicine, pH 9.0, 10 mM 2-mercaptoethanol and 1% (v/v) Triton X-100 was incubated at 0°C for 30 min. After this a 0.1 ml aliquot was diluted 1:4 with the same buffer and 0.02 ml taken for assay in 1.0 ml reaction mixtures containing 0.17 M Bicine, pH 9.0, 1.0 mM NAD^+ and 0.05 to 1.0 μM pregnenolone. (B) A 0.5 ml aliquot of solubilized microsomes was diluted with 0.1 ml of 120 mM H_2O_2 mM H_2O_2 and incubated at 0°C for 60 min then 0.02 ml aliquots were taken for assay as in (A). (C) A 0.2 ml sample of H_2O_2-treated 3β-HSD from (B) was diluted with 0.04 ml of 100 mM 2-mercaptoethanol, incubated at 0°C for 60 min and then diluted 1:3 with buffer. Aliquots of 0.02 ml were taken for assay as in (A) and (B). Units in the figure are v = nmol/ml/min and v/[pregnenolone] = nmol/ml/min/μM.

fluorometric procedure. The K_I for androstenedione (non-competitive inhibition) was less than or equal to 0.32 μM. A more sensitive radioisotopic procedure will be needed for quantitation of K_I values for this form of the enzyme.

With H_2O_2-treated microsomes progesterone (Fig. 6), androstenedione (Fig. 7) and 20α-hydroxyprogesterone (Fig. 8) were noncompetitive inhibitors. The respective K_I values of 5.5, 0.83 and 4.1 μM are in reasonable agreement with previous estimates[2, 3]. These data, obtained with the fluorometric assay, established that the dehydrogenation reaction is inhibited by these compounds.

DISCUSSION

The data presented here show that 3β-HSD can exist in at least two forms with quantitatively significant differences in kinetic properties.

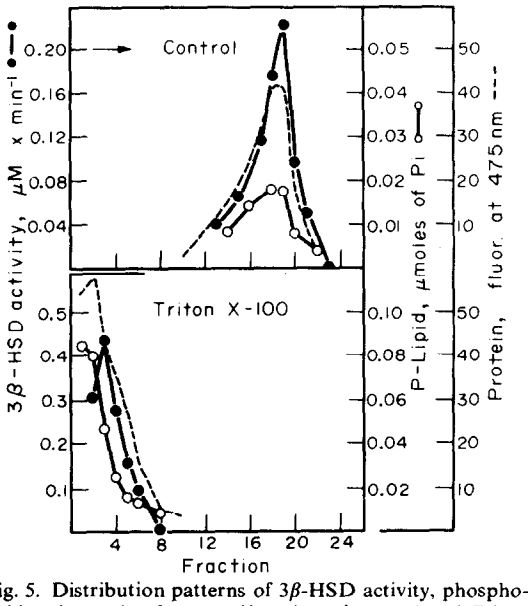

Fig. 5. Distribution patterns of 3β-HSD activity, phospholipid and protein after centrifugation of control and Triton X-100 solubilized microsomes on 10–50% (w/v) sucrose gradients. In the control gradient a 0.5 ml sample of microsomes containing 2.1 mg of protein was layered on top of a 10–50% sucrose gradient containing 0.08 M Bicine, pH 9.0 and 0.1 M 2-mercaptoethanol. In the solubilized sample a 1.0 ml suspension of microsomes containing 10.4 mg of protein, 1% Triton X-100, 7.0 mM 2-mercaptoethanol and 0.14 M Bicine, pH 9.0 was incubated at 0°C for 60 min. Subsequently a 0.5 ml aliquot was layered onto a 10–50% sucrose gradient containing 1% Triton X-100, 10 mM 2-mercaptoethanol and 0.08 M Bicine, pH 9.0. Details of centrifugation conditions are given in Methods and Materials. Assay mixtures of 1.0 ml contained 0.14 M Bicine, pH 9.0, 1.0 mM NAD^+ and 9 μM pregnenolone.

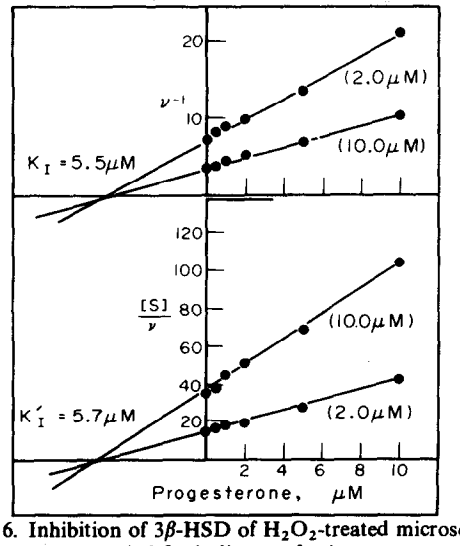

Fig. 6. Inhibition of 3β-HSD of H_2O_2-treated microsomes by progesterone. A 0.5 ml aliquot of microsomes containing 22.3 mg of protein in buffer consisting of 0.25 M sucrose, 0.10 M Bicine, pH 9.0, and 10 mM 2-mercaptoethanol was combined with 0.1 ml of 120 mM H_2O_2 and incubated at 0°C for 1 h. Subsequently 0.01 ml samples were withdrawn and assayed in 1.0 ml reaction mixtures containing 0.17 M Bicine, pH 9.0, 1.0 mM NAD^+, 0–10 μM progesterone and 2.0 or 10.0 μM pregnenolone (indicated in parentheses in the figure). Further details of fluorescence measurements are given under Methods and Materials. Units are v^{-1} = min/μM and [S]/v = min.

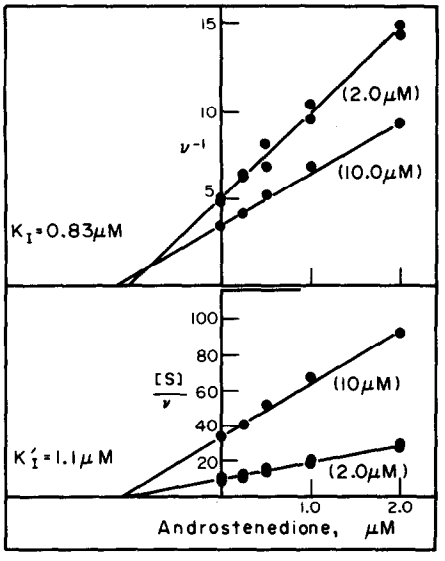

Fig. 7. Inhibition of 3β-HSD of H_2O_2-treated microsomes by androstenedione. Microsomes were incubated with H_2O_2 as given in legend to Fig. 6. Aliquots of 0.01 ml were then assayed in 1.0 ml reaction mixtures containing 0.17 M Bicine, pH 9.0, 1.0 mM NAD^+, 0 to 2.0 μM androstenedione and 2.0 or 10.0 μM pregnenolone (indicated in parentheses). Units are v^{-1} = min/μM and [S]/v = min.

Previous estimates of the K_I values for progesterone (10 μM, non-competitive) by Wiener and Allen[1, 2] and estimates of K_M values for pregnenolone of 2.8, 1 and 2 μM[5] are essentially confirmed by the experiments reported here with H_2O_2-treated enzyme. In those studies placental homogenates and microsomes were prepared in buffered 0.25 M sucrose lacking 2-mercaptoethanol, a procedure which would be expected to yield enzyme comparable to the oxidized (H_2O_2-treated) form. Similarly the K_M values for NAD^+ with H_2O_2-treated enzyme are in reasonable agreement with the value of 8 μM determined by Lübbert *et al.*[5].

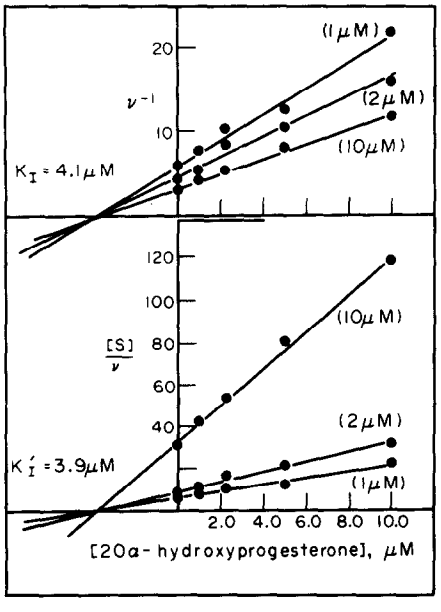

Fig. 8. Inhibition of 3β-HSD of H_2O_2-treated microsomes by 20α-hydroxyprogesterone. A 0.3 ml sample of microsomes containing 12 mg of protein in buffer composed of 0.25 M sucrose, 10 mM 2-mercaptoethanol and 0.10 M Bicine, pH 9.0, was combined with 0.06 ml of 120 mH H_2O_2 and incubated at 0°C for 30 min. Aliquots of 0.01 ml were then assayed in 1.0 ml reaction mixtures containing 0.17 M Bicine, pH 9.0, 1.0 mM NAD^+, 0 to 10 μM 20α-hydroxyprogesterone and 1, 2 or 10 μM pregnenolone. Further experimental details are given under Methods and Materials.

Since the enzyme is membrane-bound and thus could be influenced by membrane components such as phospholipids, carbohydrates or other membrane proteins, the structural bases for the effects of H_2O_2 and 2-mercaptoethanol could be complex. The observations that the K_M values for pregnenolone with detergent-solubilized 3β-HSD in both H_2O_2 and 2-mercaptoethanol-treated forms did not differ significantly from those for the membrane-bound enzyme support the conclusion that these reagents are affecting the enzyme protein *per se*.

Exposure of proteins to H_2O_2 can result in the oxidation of the side chains of methionine and cysteine to the more polar sulfoxide and sulfenic acid moieties, respectively[13–16]. Oxidations of this type in the active site of 3β-HSD would increase the polarity of the site and thus be expected to decrease the affinity for steroids. The increases in K_M values for both pregnenolone and NAD^+ after H_2O_2 treatment are consistent with such an effect and might indicate the presence of methionine and/or cysteine residues at the active site. Such residues have not been identified in 3β-HSD but affinity labeling experiments have shown the presence of cysteine residues at or near the active site of human placental 17β-hydroxysteroid dehydrogenase[17, 18]. The observations that K_M values were markedly affected but that V_{max} was the same for both H_2O_2- and-mercaptoethanol-treated 3β-HSD also suggest that substrate binding rather than the hydride-transfer step is being affected.

At present the significance of the kinetic differences for the *in vivo* activity of 3β-HSD is not known. The long recognized susceptibility of protein sulfhydryl residues to oxidation by molecular oxygen[19] would tend to favor the 2-mercaptoethanol-treated form of the enzyme as the active form. If this is the case, then the K_M for pregnenolone may be significantly lower *in vivo* than previously thought and approach the dissociation constant for the high-affinity progesterone binding sites detected in microsomes from liver and myometrium[20, 21]. That the high-affinity sites for progesterone in rat liver microsomes are enzyme active sites has been suggested[21].

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