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Affinity Labeling of Human Placental 17β-Estradiol Dehydrogenase and 20α-Hydroxysteroid Dehydrogenase with 5'-[*p*-(Fluorosulfonyl)benzoyl]adenosine†

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ABSTRACT: Two pyridine nucleotide linked oxidoreductase activities, 17β-estradiol dehydrogenase and 20α-hydroxysteroid dehydrogenase, which were copurified from human placental cytosol as a homogeneous enzyme preparation, may represent dual activity by one enzyme. The affinity labeling nucleotide analogue 5'-[*p*-(fluorosulfonyl)benzoyl]adenosine, which binds at the cofactor site as a competitive inhibitor of NADH (k_i = 1.7 mM), simultaneously and identically inactivated both the 17β and 20α activities in a time-dependent and irreversible manner following pseudo-first-order kinetics. NADH and NAD^+ markedly protected both activities from inactivation, and the substrate steroids, estrone, estradiol, progesterone, and 20α-hydroxy-4-pregnen-3-one, conferred similar protection, though less than cofactor, against simultaneous loss of both activities. Stoichiometric studies indicated that 2 mol of affinity labeling nucleotide were bound per mol of completely inactivated enzyme dimer. The coincident and identical loss of both activities under all experimental conditions is further evidence that 17β-estradiol dehydrogenase and 20α-hydrosteroid dehydrogenase in human placental cytosol represent bifunctional, stereospecific, oxidoreductase activity at one active site on a single protein.

The usefulness of the affinity labeling nucleotide analogue 5'-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSA)[1] for the study of nucleotide-dependent enzymes has been clearly established (Pal et al., 1975; Wyatt & Colman, 1977; Colman et al., 1977). Furthermore, Sweet & Samant (1980) used this reagent to confirm the hypothesis that the 3α- and 20β-oxidoreductase activities of cortisone reductase (EC 1.1.1.53) represent bifunctional enzyme activity at a single active site. We have reported purification to homogeneity of a "protein fraction" from human placental cytosol which contains both 17β-estradiol dehydrogenase and 20α-hydroxysteroid dehydrogenase activities (Strickler & Tobias, 1980). Affinity alkylation of this "protein fraction" with 16α-bromoacetoxyprogesterone suggested that 17β-estradiol dehydrogenase and 20α-hydroxysteroid dehydrogenase represent dual enzyme activity at one active site (Strickler et al., 1981). We now describe further evidence that 17β-estradiol dehydrogenase and 20α-hydroxysteroid dehydrogenase are a function of a single enzyme active site.

Experimental Procedures

Materials. Purchased reagents and their suppliers were the following: nucleotides (NAD^+ and NADH), adenosine, 5'-[*p*-(fluorosulfonyl)adenosine hydrochloride, and iodoacetic acid from Sigma Chemical Co.; *p*-(fluorosulfonyl)benzoyl chloride from Aldrich Chemical Co.; Coomassie brilliant blue G-250 from Eastman Kodak Co.; estrone, 17β-estradiol, 17α-estradiol, progesterone, 20α-hydroxy-4-pregnen-3-one, and cholesterol from Sigma Chemical Co., which were found to be chromatographically pure and to have correct melting points; deuterated acetone from Stohler Isotope Chemicals; and inorganic chemicals and reagent grade organic solvents, which were not distilled prior to use, from Fisher Scientific Co. Glass-distilled deionized water was used for all aqueous solutions. Buffer A was 0.01 M potassium phosphate buffer, pH 7.0, containing 5 mM EDTA and 20% glycerol (v/v).

Methods. Melting points were determined in an Electrothermal apparatus from Fisher Scientific Co. Infrared spectra were obtained in potassium bromide pellets by using a Beckman Acculab IR-4. Nuclear magnetic resonance spectra were measured in deuterated acetone by using a Varian T-60. Ultraviolet absorption spectra were recorded on a Varian Cary 219 spectrophotometer. Protein concentrations were determined by the Coomassie blue method (Bradford, 1976).

Enzyme Preparation. A "protein fraction" containing both 17β-estradiol dehydrogenase and 20α-hydroxysteroid de-

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[1] Abbreviations used: FSA, 5'-[*p*-(fluorosulfonyl)benzoyl]adenosine; NADH, β-nicotinamide adenine dinucleotide (reduced form); NAD^+, β-nicotinamide adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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hydrogenase activities was purified from human placenta as previously reported (Strickler & Tobias, 1980) and found to be homogeneous by $NaDodSO_4$–polyacrylamide disc gel electrophoresis. The specific activity for 17β-estradiol dehydrogenase, measured spectrophotometrically, was 7 units/mg.

Enzyme Assays. Assays for 17β-estradiol dehydrogenase activity were performed with the following solutions added to a final volume of 1.0 mL in matched 1-cm path length cuvettes: 0.775 mL of 0.05 M potassium phosphate buffer, pH 6.5, 0.1 mL of NADH in distilled water (0.14 mM) amd 0.1 mL of estrone in ethanol (0.1 mM). The reaction was initiated with 0.025 mL of enzyme incubation mixture. Assays for 20α-hydroxysteroid dehydrogenase activity used 0.6 mL of 0.05 M potassium phosphate buffer, pH 6.5, 0.1 mL of NADH in distilled water (0.14 mM), and 0.1 mL of progesterone in ethanol (0.18 mM). The reaction was initiated with 0.2 mL of enzyme incubation solution. Assays were performed in duplicate at 25 °C by using a Varian Cary 219 recording spectrophotometer. The slope of the initial linear decrease in absorbance at 340 nm (due to the oxidation of NADH) as a function of time was used to calculate enzyme activity.

Synthesis of p-(Fluorosulfonyl)benzoic Acid. p-(Fluorosulfonyl)benzoyl chloride (1 g) was dissolved in 5 mL of 1,4-dioxane to which 1.5 mL of distilled water was added dropwise. After standing at 25 °C for 45 min, the reaction was chilled on ice and the product precipitated by the addition of distilled water. After the product was dried over phosphorus pentoxide, the 850 mg of white powder had a melting point of 272–274 °C (lit. mp 271 °C; Davies & Dick, 1932): IR 3000 (OH), 1710 (O=C–OH), and 760 cm^{-1} (a peak between 755 and 815 cm^{-1} is S—F; Colthup et al, 1975); NMR, δ 8.2 (aromatic H) and 5.0 (carboxylic H).

Results

Inactivation of 17β and 20α Activities by FSA. Incubations of enzyme (2.5×10^{-6} M) in buffer A, pH 7.0, and increasing concentrations of FSA were assayed for both 17β-estradiol dehydrogenase and 20α-hydroxysteroid dehydrogenase activities. Both enzyme activities were simultaneously and identically inactivated in a time-dependent and irreversible manner following saturable pseudo-first-order kinetics (Figure 1). This family of curves was analyzed as suggested for irreversible inhibitors by Kitz & Wilson (1962):

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_3} E\text{–}I$$

A k_{app} was determined from the slopes of the ln (% activity) vs. time plots (Figure 1), and the double-reciprocal plot, $1/k_{app}$ vs. 1/[inhibitor]; was linear ($R^2 = 0.96$). From the formula

$$\frac{1}{k_{app}} = \frac{K_I}{k_3(I)} + \frac{1}{k_3}$$

the K_I was 0.68 mM and the k_3 was 1.6×10^{-3} s^{-1}.

Incubations performed in buffer, identical with buffer A except pH 6.1 and a 400 μM concentration of FSA, again demonstrated linear, time-dependent, simultaneous inactivation of both enzyme activities. Whereas the $t_{1/2}$ of inactivation at this FSA concentration in pH 7.0 buffer was 17 min (Figure 1), the $t_{1/2}$ at pH 6.1 was 186 min.

For evaluation of the stability of FSA (1.14×10^{-3} M), it was incubated in buffer A containing 10% ethylene glycol. A fluoride ion electrode, calibrated against potassium fluoride in the same buffer (1×10^{-1}–1×10^{-5} M fluoride), was used to monitor the rate of fluoride release from the reagent. The obsesrved first-order rate constant for fluoride release in pH

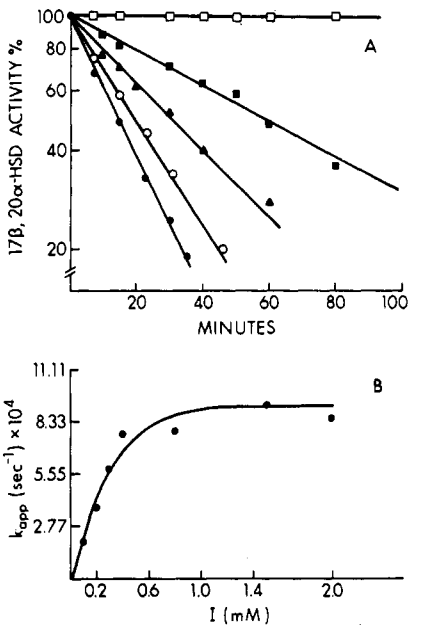

FIGURE 1: Inactivation of the 17β-estradiol dehydrogenase and 20α-hydroxysteroid dehydrogenase activities by various concentrations of 5′-[p-(fluorosulfonyl)benzoyl]adenosine. (A) Enzyme (2 μM) in 6 mL of buffer A was incubated at 25 °C with inactivator [final concentrations (■) 100 μM; (▲) 200 μM, (○) 300 μM and (●) 400 μM] in 0.12 mL of ethylene glycol. Identical control incubations contained adenosine. At the indicated times, aliquots were removed and assayed for both 17β and 20α activities (Methods). The percentage of enzyme activity is a logarithmic scale along the ordinate, and time is a linear scale along the abcissa. For simplification of the graphic presentation, the single points represent the mean of duplicate assays for both activities. (B) The date in (A) as well as inactivation studies using saturating concentrations of inactivator (500–2000 μM) were used to calculate the k_{app} ($0.693/t_{1/2}$) and construct the plot demonstrating saturation kinetics.

7.0 buffer, 0.0124 min^{-1}, was slightly slower than reported by Likos et al. (1980) using buffer pH 8.6 and 70-fold slower than the k_3 for the enzyme inactivation. Furthermore, when the mathematical formulation derived by Likos et al. (1980) was used to correct for this small loss of fluoride during the time course of inactivation, the observed first-order rate constant, 0.046 min^{-1}, using a half-saturating concentation of FSA (6.78×10^{-4} M) was insignificantly different from the calculated second-order rate constant, 0.047 min^{-1} mM^{-1}. Thus, reagent deterioration did not significantly affect the initial linear inactivation kinetics.

Since FSA contains an ester bond between the affinity moiety, adenosine, and the reactive [p-(fluorosulfonyl)benzoyl group, hydrolysis of this linkage could release a nonspecific labeling reagent. For assessment of the functional significance of this possibility, enzyme (2×10^{-7} M) in buffer A was incubated with p-(fluorosulfonyl)benzoic acid (5×10^{-4} M). Very slow inactivation ($t_{1/2}$ = 11.6 h compared to $t_{1/2}$ 15 min for an identical incubation containing FSA) allowed calculation of an observed first-order rate constant (0.059 h^{-1}) which was $^1/_{42}$ the observed first-order rate constant for FSA in an identical incubation. Thus, nonspecific enzyme inactivation was not a significant component in these studies.

Evidence That FSA is an Active-Site-Directed Reagent. Since FSA lacks the nicotinamide moiety, which effects the "hydrogen" transfer during oxidation and reduction, it cannot act as a nucleotide substrate. Thus, kinetic studies were performed to show that FSA competitively inhibits NADH-linked oxidation. Assay mixtures contained 1×10^{-4} M estrone in 0.1 mL of ethanol, 0.125×10^{-3}–1×10^{-3} M FSA (inhibitor) in 0.1 mL of ethylene glycol, and 0.05 M potassium

Table I: Protection Effect of Steroid and Nucleotide Substrates on Enzyme Inactivation by 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine[a]

protector (concn)[b]	activity at 30 min (%)
none	25
NAD^+ (400 μM)	74
NADH (100 μM)	70
17β-estradiol (30 μM)	50
17β-estradiol (10 μM)	44
estrone (10 μM)	37
progesterone (500 μM)	34
20α-hydroxy-4-pregnen-3-one (150 μM)	32
17α-estradiol (10 μM)	38
cholesterol (50 μM)	25

[a] Enzyme (2.5×10^{-6} M) was incubated with FSA (400 μM) at 25 °C in buffer A containing 4% ethylene glycol. After 30 min, assays were performed for both 17β-estradiol dehydrogenase and 20α-hydroxysteroid dehydrogenase activities as described under Methods. [b] The concentration of the protector approximates its K_m or is the maximum which remains in solution.

phosphate buffer, pH 6.5, to a final volume of 1.0 mL. The reaction was initiated with 2.7 μg of enzyme in 0.1 mL of buffer A. Converging lines in the resulting Dixon plot (Dixon, 1953) indicated that FSA was a competitive inhibitor with a k_i of 1.7×10^{-3} M.

For a demonstration that the usual substrates for both 17β-estradiol dehydrogenase and 20α-hydroxysteroid dehydrogenase will protect against inactivation by FSA, incubations of enzyme (2.5×10^{-6} M) and FSA (400 μM) in buffer A containing 4% ethylene glycol were studied with the addition of steroids or cofactor as protectors (Table I). The greatest protection against inactivation was observed when nucleotides were present in the incubation, but a smaller protective effect from all substrate steroids was seen. Cholesterol, which does not bind at the active site, gave no protection. 17α-Estradiol was protective because it is an excellent inhibitor for estrone reduction (Dixon analysis, k_i = 13 μM).

So that it could be further shown that a single binding site is being utilized for both the 17β- and 20α-oxidoreduction, kinetic studies were performed to show that progesterone competitively inhibits enzymatic oxidation of 17β-estradiol. Solutions contained 1.5×10^{-4} M NAD^+ in 0.1 mL of distilled water, 3×10^{-6}–1×10^{-5} M 17β-estradiol in 0.1 mL of ethanol, and 7.5×10^{-5}–1×10^{-3} M inhibitor (progesterone) in 0.1 mL of propylene glycol, in 0.05 M sodium carbonate buffer, pH 9.2, to a final volume of 1.0 mL. The reaction was initiated with 2.7 μg of enzyme in 0.01 mL of buffer A. Converging lines in a Dixon (1953) analysis indicated that progesterone was a competitive inhibitor, $k_i = 2.9 \times 10^{-4}$ M.

Stoichiometry of Enzyme Inactivation by FSA. Enzyme (2.5×10^{-6} M) was incubated with FSA (6.8×10^{-4} M) in 5.9 mL of buffer A containing 6% ethylene glycol. When enzyme assays showed 50% activity and complete (93%) inactivation, 2.5-mL aliquots were removed, the affinity-labeling reaction was quenched with 142 μmol of 2-mercaptoethanol, and the solution was dialyzed against 0.005 M potassium buffer, pH 7.0, containing 2.5 mM EDTA. During the 2 h after addition of 2-mercaptoethanol, enzyme reactivation, as was reported for pyruvate kinase (Likos & Colman, 1981), was not observed. During a 24-h dialysis, the 2-L bath was changed 4 times. A control incubation containing enzyme alone was treated identically. The protein concentration of the dialyzed enzyme solutions was determined, and identical quantities were used in the ultraviolet absorption spectral analysis (Figure 2). The number of moles of FSA incorporated per mole of inactivated enzyme protein was calculated from the difference in the absorbance at 259 nm between the affinity-labeled and native enzymes, using the extinction coefficient of free FSA at that wavelength (Pal et al., 1975). The stoichiometric analysis shows that 2 mol of FSA is incorporated per mol of enzyme dimer (m_r 68 000).

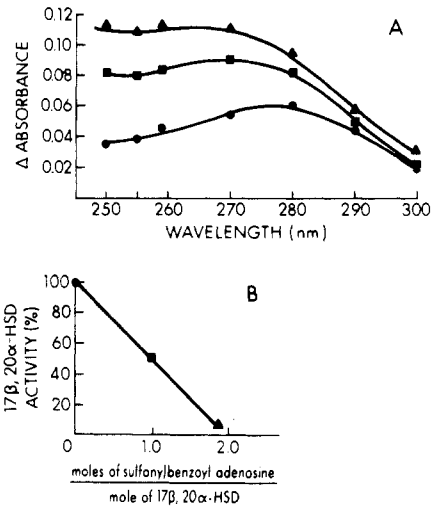

FIGURE 2: Stoichiometry of incorporation of 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine, correlated with the decrease of 17β and 20α activities. (A, representative profiles) The ultraviolet adsorption spectra of native enzyme (●), 50% inactivated enzyme (■), and 93% inactivated enzyme (▲) were used to calculate the incorporation of affinity label on the basis of FSA λ_{max}^{EtOH} at 259 nm (=1.58×10^4 cm^{-1} M^{-1}) and enzyme (2.5 μM) dimer M_r 68 000. (B) The percentage of 17β and 20α activity is plotted along the ordinate and moles of (*p*-sulfonylbenzoyl)adenosine covalently bound per mole of enzyme are along the abscissa (r^2 = 0.99; y = 100.6, x = 2.01). The results are the mean of duplicate experiments.

Discussion

The nucleotide analogue 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine has been used to probe the cofactor binding region of bovine liver glutamate dehydrogenase (Pal et al., 1975), rabbit muscle pyruvate kinase (Wyatt & Colman, 1977), the β subunit of mitochondrial F_1-ATPase (Esch & Allison, 1978), the catalytic subunit of cAMP-dependent protein kinase (Zoller & Taylor, 1979), the bifunctional oxidoreductase 3α,20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* (Sweet & Samant, 1980), *Escherichia coli* glutamine synthetase (Foster et al., 1981), bovine heart muscle cAMP-dependent protein kinase (Hixson & Krebs, 1979), and yeast pyruvate kinase (Likos et al., 1980). It is not surprising that this reagent affinity labels the nucleotide binding region of the soluble 17β-estradiol dehydrogenase/20α-hydoxysteroid dehydrogenase in human placental cytosol.

FSA inactivates both the 17β and 20α activities in a time-dependent and irreversible manner following saturable, pseudo-first-order kinetics. The evidence that this reagent selectively occupies the nucleotide binding region is the following: (1) Kinetic studies analyzed according to Dixon (1953) show competitive binding between FSA and NADH. (2) Inactivation curves at several concentrations of FSA conform to the model for irreversible active-site inhibitors formulated by Kitz & Wilson (1962). (3) The rate of enzyme inactivation is markedly slowed in the presence of cofactor, consistent with competition at the same site. Directed binding of FSA is also inferred by the studies using *p*-(fluorosulfonyl)benzoic acid. When the reagent lacks the adenosine moiety which presumably confers on FSA a structural similarity to the pyridine nucleotides, inactivation is comparatively

negligible. Wyatt & Colman (1977) observed similar very slow inactivation of rabbit muscle pyruvate kinase by *p*-(fluorosulfonyl)benzoic acid.

The protection observed when steroid hormones, capable of binding at the active site, are coincubated with enzyme and FSA is analogous to the protection which nucleotide substrates offer during inactivation by affinity alkylating steroid derivatives (Pons et al., 1976). This crossover of protection between cofactor and substrate is consistent with the model of the active site of 17β-estradiol dehydrogenase proposed by the French investigators (Pons et al., 1976). They envision the active site as a cul-de-sac which simultaneously accepts and aligns both components of the oxidoreductase reaction. Furthermore, Chin et al. (1980) suggest that within the active site of 17β-estradiol dehydrogenase, the steroid and the nucleotide are aligned side by side, with the adenosine moiety proximating the steroid A ring. Assuming such an alignment when FSA and steroid substrate simultaneously occupy the active center, the *p*-(fluorosulfonyl)benzoyl group would be relatively hindered for nucleophilic attack on an active-site amino acid, and a slower rate of enzyme inactivation would be observed.

The stoichiometry of enzyme inactivation shows that 2 mol of FSA is covalently bound per mol of completely inactivated enzyme dimer. We have shown (Strickler et al., 1981) that the affinity alkylating steroid, 16α-bromoacetoxyprogesterone, inactivates an identical enzyme preparation, and the stoichiometry is 2 because this reagent dialkylates a histidine within the enzyme active site. Thus, a similar dialkylation of a single amino acid could account for this stoichiometry. It is equally possible that the *p*-(fluorosulfonyl)benzoyl group has access to two equally reactive amino acid residues. Second, Warren & Crist (1967) observed promotion of NAD^+ reduction by 17β-estradiol dehydrogenase at nucleotide concentrations above 16.6 μM. They postulated that a second nucleotide binding site would explain their observation. Since the stoichiometry experiments used a nucleotide analogue concentation of 680 μM, this postulated second binding region might account for the observed value of 2. Finally, it is possible that a specific active-site-directed labeling by FSA is associated with a second nonspecific reaction. However, since the enzyme is minimally susceptible to nonspecific inactivation by *p*-(fluorosulfonyl)benzoic acid and completely resistant to the nondirected alkylator iodoacetic acid (unpublished data), this explanation seems the least likely.

The second aim of these investigations was to test the hypothesis that 17β-estradiol dehydrogenase and 20α-hydroxysteroid dehydrogenase activities in human term placental cytosol represent bifunctional enzyme activity on one protein. This hypothesis was first stated by Purdy et al. (1964) and is supported by our observations that these activities copurify (Strickler & Tobias, 1980) and are identically inactivated by the affinity alkylating steroid 16α-bromoacetoxyprogesterone (Strickler et al., 1981). In these studies, both the 17β–estradiol dehydrogenase and 20α-hydroxysteroid dehydrogenase activities were simultaneously and identically inactivated by several concentrations of FSA in buffers of different pH. During the protection experiments, identical slowing of the rate of inactivation was observed for both 17β and 20α activities irrespective of whether the protector was an estrogen, a progestin, or a nucleotide cofactor. The protective value of the estrogens and the progestins was similar, and the nonsubstrate steroid cholesterol failed to influence either activity. Finally, kinetic studies show that progesterone is a competitive inhibitor for 17β-estradiol oxidation.

Although it may be argued that two oxidoreductases which use the same pyridine nucleotide cofactors should be similarly inactivated by an affinity labeling cofactor analogue, identical behavior under all experimental conditions would seem unlikely. Second, when these data are coupled with an identical result obtained from affinity alkylating steroid studies, the argument of chance occurrence is substantially weakened. Finally, when these observations are framed by the reports that the 20α-hydroxysteroid dehydrogenase and 17β-estradiol dehydrogenase activities in rabbit ovary (Rodway & Rahman, 1978) and in human endometrium (Tseng & Gurpide, 1979) may arise from one enzyme, a compelling collage argues that human placental 17β-estradiol dehydrogenase and 20α-hydroxysteroid dehydrogenase represent bifunctional oxidoreductase activity at the active site of one enzme.

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