Vol. 83, No. 1, 1978
July 14, 1978

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 187-194

SUBSTRATE CHARACTERISTICS OF PROGESTERONE-ALBUMIN CONJUGATES WITH 20β-HYDROXYSTEROID DEHYDROGENASE

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Received May 1,1978

SUMMARY: Progesterone covalently conjugated to bovine serum albumin through the 21-position is a substrate for 20β-hydroxysteroid dehydrogenase(E.C.1.1.1.53) from *S. hydrogenans*. Varying the progesterone to albumin molar ratio in a range of 1:1 to 19:1 results in variations in the apparent Km and Vmax values. A maximum in substrate activity is obtained at a progesterone to albumin ratio of 6:1, while at either extreme in the range of these ratios the activity is a minimum. Progesterone-albumin conjugates attached at the steroid 2α-, 6β, or 11α-position in varying ratios did not produce measurable substrate activity. The results show that a steroid need not be free in solution to serve as a substrate for the steroid oxido-reductase.

A recent report mentioned that although estrone was covalently attached to the active site of human placental estradiol 17β-dehydrogenase, following an affinity labeling reaction, it could serve as a substrate for the native form of the enzyme (1). This implied that a steroid need not be free in solution in order to serve as a substrate for a steroid oxido-reductase. It seemed desirable to systematically explore the general nature of this phenomenon by studying the activity of a variety of steroid-protein covalent conjugates with a steroid oxido-reductase. The present report describes the synthesis of a series of hemisuccinyloxyprogesterone-bovine serum albumin conjugates in which the steroid to protein ratios were varied (2). The substrate characteristics of these conjugates with 20β-hydroxysteroid dehydrogenase (E.C.1.1.1.53) from Streptomyces hydrogenans were determined. The implications of substrate activity obtained with a steroid containing albumin

0006-291X/78/0831-0187$01.00/0

as a macromolecular substituent are discussed.

MATERIALS AND METHODS: Progesterone, 6β-hydroxyprogesterone, 11α-hydroxyprogesterone, and deoxycorticosterone from Steraloids Co., were useable without further purification. Nucleotides (NAD^+, NADH), and crystalline bovine serum albumin Cohn fraction V were purchased from Sigma Chemical Co. 20β-Hydroxysteroid dehydrogenase (E.C.1.1.1.53) from *Streptomyces hydrogenasis*, (Boehringer Mannheim Co.) with specific activity of 18 U/mg was suitably homogeneous for use without further purification. iso-Butylchloroformate (Aldrich Chemical Co.) was freshly distilled over anhydrous calcium chloride prior to its use. Tri-n-butylamine, succinic anhydride, dioxane, and other organic solvents (Fisher Scientific Co.) were distilled before use. Union Carbide dialysis tubes (Scientific Products) were used for performing dislyses.

Synthesis of hemisuccinyloxyprogesterone-bovine serum albumin conjugates. The bovine serum albumin conjugated progesterone derivatives were prepared by modification of a method described by Erlanger, et al (3). The desired molar ratio of steroid to albumin was obtained by adjusting the ratio of the empirically optimized reaction mixture. Accordingly, a 0.200 ml aliquot of a mixed anhydride solution, prepared from 135 mg (310 μmole) of 21-hemisuccinyloxyprogesterone, 40 mg (330 μmole of freshly distilled isobutyl chloroformate, and 75 mg (330 μmole tri-n-butylamine, in 3 ml of anhydrous dixane), was added to a solution containing 430 mg (6.5 μmole) of crystalline bovine serum in 22 ml of 50% dixane-0.05M phosphate buffer pH 7.5 at 10°C, then stirred at 25°C for 4 hrs. The reaction mixture was dialysed against four changes of 3 L of water, adjusted to pH 7.0 with 0.2N NaOH. The retentate was centrifuged at 15,000 xg for 1 hour at 15°C, and the supernatant was lyophilized. The steroid-protein conjugate was dried under vacuum over phosphorous pentoxide to give 350 mg of product.

Weighed samples of the conjugate were dissolved in 0.05M phosphate buffer, pH 7.0, and spectrophotometrically quantitated. The molar ratio of progesterone:albumin in this material was 2:1. The above conjugation reaction reproducibly gave this ratio. In order to obtain different molar ratios of progesterone:albumin an appropriate volume of the mixed anhydride solution was added to 430 mg of albumin in 50% dioxane-buffer. It was most convenient to prepare the above solutions of mixed anhydride and albumin in bulk, divide the albumin solution into several 22 ml portions, and to add appropriate volumes of mixed anhydride to each portion. The resulting reaction mixtures could be simultaneously incubated, dialysed, and lyophilized.

Determination of progesterone:albumin molar ratio in the conjugates. The progesterone-albumin conjugate or crystalline albumin was accurately weighed (0.005 mg to 1.000 mg), dissolved on 3.00 of 0.05M phosphate buffer pH 7.0, and the resulting solution was transferred to matched 1 cm path length quartz cuvettes. The albumin solution was placed in the reference cuvette. The O.D at 240 nm due to the progesterone-albumin conjugate solution was recorded, and the molar ratio of steroid to protein derived from a standard curve previously obtained from solutions of 21-hemisuccinyloxyprogesterone and albumin mixtures. These values were further confirmed by adding known quantities of hemisuccinyloxyprogesterone in 0.05M phosphate buffer pH 7.0 to the albumin reference cuvette, until the absorbance due to the progesterone-albumin conjugate solution in the sample cuvette was suppressed to nearly 0.000 (the same volume of buffer was added to the sample cuvette). Results from each method agreed to within 2-3%.

Enzyme kinetic studies. Enzyme assays were conducted with the following solutions added to a final volume of 3.0 ml in matched 1cm x 1cm cuvettes:

2.8 ml of 0.05M potassium phosphate buffer, pH 6.5, containing varying amounts of hemisuccinyloxyprogesterone-bovine serum albumin conjugates; 0.100 ml aliquot of a solution containing 25 μg to 75 μg of 20β-hydroxysteroid dehydrogenase in 0.05M phosphate buffer, pH 7.0; 0.100 ml of NADH (0.1 μmole) in 0.05M phosphate buffer, pH 7.0. The slope of the initial linear decrease in absorbance at 340 nm (due to oxidation of NADH) as a function of time was used to calculate enzyme activity. Substrate concentrations were taken as the nominal steroid concentration resulting from solution of a weighed amount of progesterone-albumin conjugate containing the measured steroid to protein ratio, as described above. Assays performed in triplicate were conducted at 25 ± 1°C in a Beckman model 25 recording spectrophotometer. Kinetic data were fitted by least mean squares with a Hewlett-Packard model 97 programmable calculator. Lineweaver-Burke plots of the kinetic data provided the apparent KM and Vmax values (summarized in Table 1).

RESULTS AND DISCUSSION: 6β-, 11α, and 21-Hemisuccinyloxyprogesterone-bovine serum albumin conjugates were synthesized by reacting the mixed anhydride obtained from iso-butylchloroformate and the appropriate hemisuccinyloxyprogesterone, with crystalline bovine serum albumin in 50% dioxane-aqueous 0.05M phosphate pH 7.5 buffer solution, at 5°C to 25°C. Following completion of the reaction the products were exhaustively dialysed against distilled water to remove unconjugated steroid, and then lyophilized. Reaction conditions and molar proportions of the reactants were empirically optimized to produce a series of conjugates with nearly integral variations in the progesterone:albumin molar ratios. The progesterone covalently bound to albumin was quantitated by comparing the optical density at 240 nm of solutions of weighed masses of the conjugates with standard solutions containing varying amounts of the appropriate hemisuccinyloxyprogesterone mixed with a similar mass of albumin.

Enzyme kinetic data were obtained with solutions of progesterone-albumin conjugates and 20β-hydroxysteroid dehydrogenase that contained the appropriate *nominal* steroid concentrations. To be certain that the kinetic data solely reflected 20β-hydroxysteroid dehydrogenase acting on the progesterone-albumin conjugates, and not 21-hydroxyprogesterone released from albumin by partial hydrolysis of the steroid ester linkage the following experiments were carried out. Solutions (5 ml) containing a progesterone-albumin conjugate (10^{-4}M in progesterone), and also a mixture of 21-hydroxyprogesterone (10^{-4}M)

TABLE 1. KINETIC DATA FROM 20β-HYDROXYSTEROID DEHYDROGENASE AND PROGESTERONE-ALBUMIN CONJUGATES

Substrate[a]	Progesterone/albumin molar ratio	Km value (M x 10^4)	Vmax (nmoles/min/μg of enzyme)
21-Hemisuccinyloxy-progesterone-bovine serum albumin	1	7.32	8.32
	2	4.01	3.24
	6	1.20	2.74
	12	18.20	24.5
	19	117.00	48.6
Progesterone		0.0395	11.58
21-Hydroxyprogesterone		0.251	3.09
21-Hemisuccinyloxy-progesterone		14.3	0.50
21-Acetoxyprogesterone		22.3	0.065
11α-Hemisuccinyloxy-progesterone		21.9	1.74

[a]Data from enzyme kinetic studies conducted with steroids not conjugated to albumin was obtained from assays performed under similar conditions (see *enzyme kinetic studies,* above) except that the buffer contained 5% to 10% ethanol.

and albumin, were assayed for substrate activity. Powdered activated charcoal (7 mg) was mixed with each of the two solutions and then they were centrifuged at 4,000 rpm for 5 min. Enzyme assays of the supernatants showed that the progesterone-albumin conjugate solution retained all of its original activity. The supernatant from the solution containing a mixture of hydroxyprogesterone and albumin had no detectable activity. Therefore, the enzyme kinetics obtained with the progesterone-albumin conjugates were due entirely to covalently bound progesterone.

21-Hemisuccinyloxyprogesterone-albumin is a substrate for 20β-hydroxysteroid dehydrogenase with apparent Km values ranging from 1.2×10^{-4}M to 1.17×10^{-2}M, and Vmax values of 2.74 nmoles/min/μg to 48.6 nmoles/min/μg of enzyme, respectively (Table 1). The enzyme kinetic constants of the conjugates depended upon both the position of attachment on the steroid, and on the ratio of progesterone to albumin in the conjugates. A plot of the apparent KM-values for 21-hemisuccinyloxyprogesterone-albumin versus the progesterone:albumin molar ratios produces a curve with maximum activity at 6:1 (Fig. 1). Experiments conducted with 6β-hemisuccinyloxyprogesterone-albumin conjugates did not result in measurable substrate activity. The 11 -conjugated steroid possessed detectable substrate activity, but it was not sufficient to provide precise kinetic data.

Substrate activity, taken as the apparent Km value, is dependent upon the molar ratio of progesterone to albumin in the 21-hemisuccinyloxyprogesterone-albumin conjugates (Fig. 1). Each albumin molecule contains approximately 60 different positions at which 21-hemisuccinyloxyprogesterone can be bound, and therefore the present data probably represents binding effects averaged over a heterogeneous substrate population. Increasing the ratio of steroid to albumin may result in the more active steroid ligands "diluted" by less active forms, or creation of new active ligands. Moreover, as the ratio of steroid to albumin varies in the conjugates, the regions on albumin that make positive contributions to binding of the progesterone moiety to the enzyme active site may become masked by the additional steroid molecules. In any case the present kinetic data shows that the substrate activity of the albumin-conjugated progesterone is very much influenced by the ratio of steroid to albumin in the conjugate.

Affinity labeling studies of 20β-hydroxysteroid dehydrogenase earlier led us to the conclusion that progesterone derivatives possess less than 1.5Å of motion in any direction during their residency at the enzyme active site (4). However, comparison of Km values from the progesterone-albumin

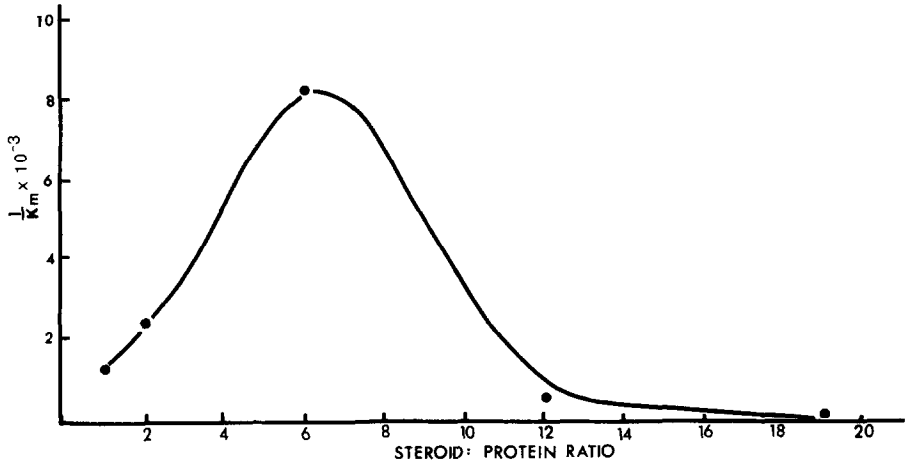

FIGURE 1. Substrate activity of 21-hemisuccinyloxyprogesterone-bovine serum albumin conjugates with 20β-hydroxysteroid dehydrogenase, correlated with progesterone:albumin ratio. A plot of the reciprocal of the apparent Km values (from Table 1) against the progesterone:albumin ratio reveals a pattern of substrate activity which peaks at a 6:1 ratio. The inverse relationship between the Michaelis constant Km, and the catalytic rate constant, $k_{cat.}$, for a *family* of substrates has been previously shown (9). The $k_{cat.}$ is a measure of substrate activity, therefore 1/Km is used here as a measure of substrate activity for the family of 21-hemisuccinyloxyprogesterone-albumin conjugates.

conjugates with those from 21-acetoxyprogesterone and 21-hemisuccinyloxyprogesterone suggests that there is apparently little or no steric interference with substrate binding by the presence of the 66,000 dalton albumin substituent on progesterone (Table 1). The present results show that the steroid binding site of 20β-hydroxysteroid dehydrogenase is sufficiently flexibile to accomodate a progesterone derivative containing a macromolecular substituent, consistent with the flexible enzyme, or induced fit model of substrate-enzyme binding (5). These results also show that a steroid need not be free in solution to serve as a substrate for an enzyme. Results from previous affinity labeling experiments (4,6) are combined with the present findings in the schematic representation in Fig. 2, which depicts binding of 21-hemisuccinyloxyprogesterone-albumin at the active site of 20β-hydroxysteroid dehydrogenase.

CONCLUSION: It is well-known that steroid specific enzymes can be bound by

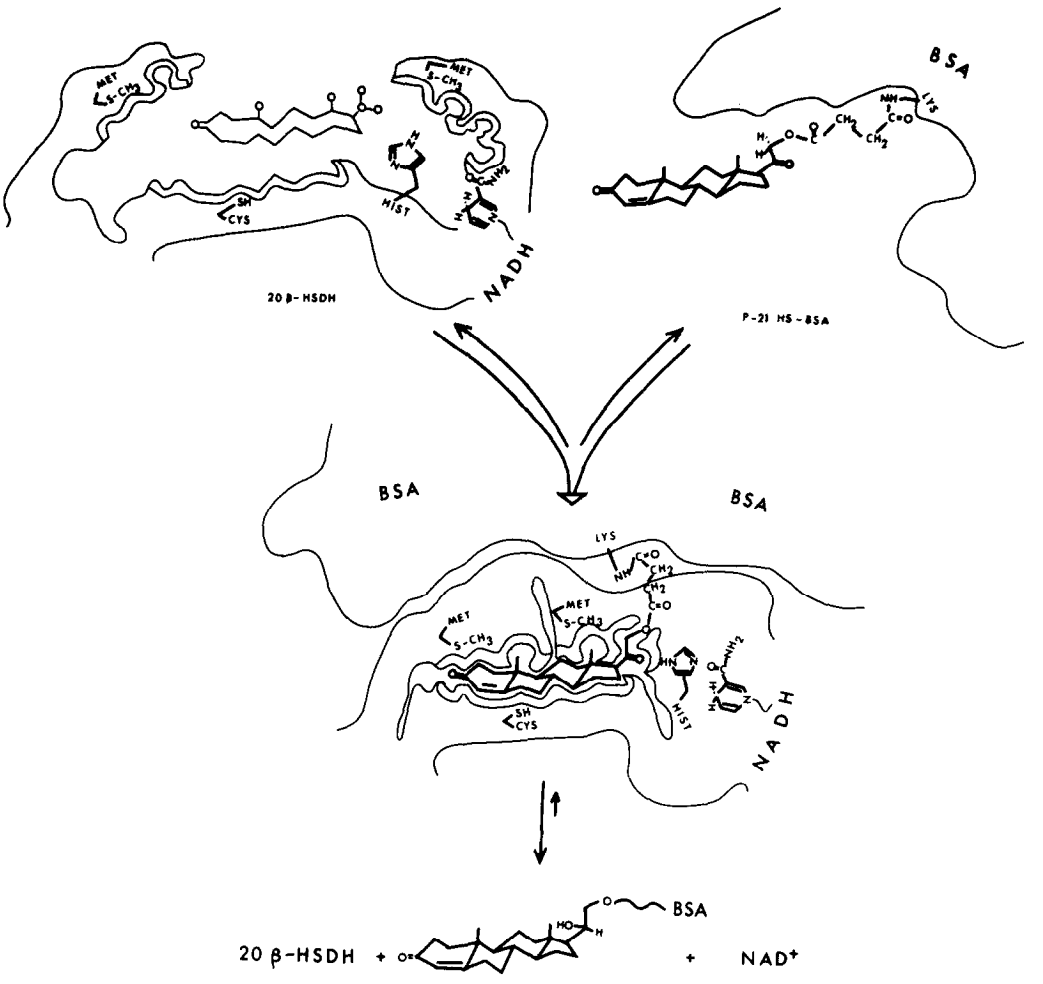

FIGURE 2. Schematic illustration of 21-hemisuccinyloxyprogesterone-bovine serum albumin conjugate binding at the active site of 20β-hydroxysteroid dehydrogenase. As the substrate (P-21 HS-BSA) approaches the steroid-binding region of the enzyme (20β-HSDH), interaction between the progesterone moiety and active site amino acids stabilizes the steroid-enzyme complex. Identity and topography of the amino acids represented in the drawing of the active site of 20β-HSDH were determined by affinity labeling experiments (4,6).

steroids which are covalently attached to an insoluble, macromolecular agrose matrix (7). This is the basis of affinity chromatography. In the present study the steroids were covalently attached to water soluble albumin which permitted us to quantitatively study the enzyme activity of the resulting progesterone conjugates. The protein-steroid-protein interactions described here suggest that a considerable range of size and aggregate charge can be

associated with a progesterone "side chain" with retention of steroid binding at an enzyme active site. This is a general phenomenon, observed with human placental 17β-hydroxysteroid dehydrogenase (1), and also with binding of progesterone-albumin and estradiol-albumin conjugates to the appropriate uterine cytoplasmic receptor proteins (8). Further investigations with these systems are expected to provide additional insight into the nature of steroid-protein interactions.

ACKNOWLEDGEMENTS: F. Sweet is recipient of a U.S. Public Health Service Research Career Development Award, HD-70788. This work was supported by grants AM-16854, HD-08235, and training grant HD-00379 from National Institutes of Health. The technical assistance of Mr. Lawrence Fink is gratefully acknowledged.

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