ENZYMIC SYNTHESIS OF STEROID SULFATES XVI. SPECIFICITY AND REGULATION OF HUMAN ADRENAL HYDROXYSTEROID SULFOTRANSFERASE

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ABSTRACT

Pure hydroxysteroid sulfotransferase (EC 2.8.2.2) of human adrenal glands possesses a wide substrate specificity towards steroids. This wide specificity has now been found to extend to simple alcohols; normal aliphatic alcohols from C_3 onwards acting as substrates with C_9 showing the highest rate. Increased rate was accompanied by a decrease in Km. In marked contrast to the sulfurylation of steroids such as dehydroepiandrosterone, which exhibit wave-like kinetics, the kinetics with simple alcohols were of the normal Michaelis-Menten type. By means of enzyme antibody and enzyme stability studies evidence was provided that one and the same enzyme was responsible for sulfurylation of hydroxyls on the 3- and 17- positions of steroids and simple alcohols. The data lend support to previous evidence that the enzyme controls the secretion of dehydroepiandrosterone sulfate via steroid-specific binding sites, enabling self-regulation in response to ACTH action.

INTRODUCTION

Dehydroepiandrosterone sulfate is secreted by the human adrenal gland in amounts of approximately 15 mg/day, making it along with cortisol quantitatively the most important secretory product of the gland. The enzyme responsible for the sulfurylation of dehydroepiandrosterone has been isolated from human adrenals in pure form (1). It exists as a dimer of identical subunits of molecular weight 34 000 and exhibits unusual wave-like kinetics. Although isolated as a single protein species, as demonstrated by acrylamide gel electrophoresis in the absence and presence of sodium dodecyl sulfate, the enzyme catalyses the sulfurylation of hydroxyl groups on a wide range of C_{18}, C_{19}

and C_{21} steroids to produce monosulfurylated derivatives (2,3). The explanation for this phenomenon is believed to reside in the ability of the steroid to approach the sulfurylation site in either of two ways; via ring A in which 3β-hydroxyl groups are sulfurylated at a greater rate than 3α-hydroxyls, or alternatively via ring D after the molecule is turned through 180^{o}. In this latter presentation, 17α-hydroxyl groups are then sulfurylated at a greater rate than 17β-hydroxyls (3).

Kinetic properties of the enzyme, examined at physiological concentrations of dehydroepiandrosterone, suggest that the enzyme is involved in regulating dehydroepiandrosterone sulfate formation via dehydroepiandrosterone allosteric sites (3). In contrast to the human adrenal hydroxysteroid sulfotransferase, the purified rat liver enzyme exhibits normal Michaelis-Menten kinetics towards steroid substrates and has recently been reported to sulfurylate simple alcohols (4). We have now examined the activity of the human adrenal enzyme towards an homologous series of normal aliphatic alcohols. Here we report that alcohols of chain length C_3 or greater are sulfurylated, with maximum activity achieved with C_9, and that the kinetics are classical Michaelis-Menten. Evidence is provided that alcohol and hydroxysteroid sulfotransferase activities reside in the same enzyme which supports the hypothesis for the presence of steroid-specific regulatory sites.

MATERIALS AND METHODS

Chemicals. The lower aliphatic alcohols were of laboratory reagent grade (British Drug Houses) and the higher alcohols, from n-hexanol onward, were obtained from Polyscience Corp., Illinois. 1,9-Nonanediol was obtained from Tokyo Kasei Kogyo Co. Ltd. Dehydroepiandrosterone and 17-epitestosterone were Sigma products. Protein A Sepharose CL-4B was obtained from Pharmacia (Australia) Pty. Ltd.

Human adrenal hydroxysteroid sulfotransferase was isolated by affinity chromatography and assayed with 3'-phosphoadenosine-5'-phospho-^{35}S-sulfate, as described previously (1). Substrates (aliphatic alcohols or steroids) were added from stock solutions in propylene glycol unless stated otherwise. In the kinetic experiments, incubations were carried out for 10 min when the reaction rate was linear. Any departures from the usual are mentioned in the figure legends.

Antibodies to the human enzyme were raised in rabbits and the IgG fraction isolated from pooled serum by chromatography on DEAE-cellulose (5).

Isolation of bovine liver steroid alcohol sulfotransferase. The same affinity chromatography system employing dehydroepiandrosterone-17-(O-carboxymethyl)-oxime linked to AH Sepharose-4B (1), was used for isolating the bovine liver enzyme. Briefly, bovine liver (200 g) was homogenised in 3 vol 0.05 M Tris-HCl buffer pH 7.5, containing 0.1 mM dithiothreitol and 0.08 M KCl. After centrifuging at 50 000 x g for 75 min, an $(NH_4)_2SO_4$ fraction precipitating between 35% and 55% saturation was prepared, and dissolved in 100 ml of 0.05 M sodium phosphate buffer, pH 7.5, containing 0.1 mM dithiothreitol. Reaction with, and elution from, the affinity gel was carried out as for the preparation of the human adrenal enzyme (1). The concentrated, dialysed eluate, when examined by 5.6% polyacrylamide gel electrophoresis, showed the presence of two major protein bands. Enzyme activity, employing dehydroepiandrosterone as sulphate acceptor (1), was associated with the less mobile protein band. The gel eluate was then chromatographed on a 25 cm x 2 cm column of DEAE-Sephadex A-50 employing a linear gradient of 0.06 M-0.30 M NaCl in 0.025 M Tris-HCl, pH 8.0, containing 1 mM β-mercaptoethanol. This step removed the contaminating protein, as verified by acrylamide gel electrophoresis. Enzymically active fractions (dehydroepiandrosterone as substrate) were pooled and used for specificity and kinetic experiments.

RESULTS

Sulfurylation of aliphatic alcohols

Estrogen sulfotransferase (EC 2.8.2.4) isolated from bovine adrenal glands or bovine placental tissue will sulfurylate natural and synthetic phenolic estrogens on the phenolic groups (6). Phenols carrying a lipophilic side chain in the p-position are also sulfurylated (7) but hydroxyl groups on steroids are not sulfurylated (8). Hydroxysteroid sulfotransferase isolated from rat liver (EC 2.8.2.2), shows a somewhat parallel behaviour in being able to sulfurylate aliphatic alcohols, in addition to a wide variety of steroids (4).

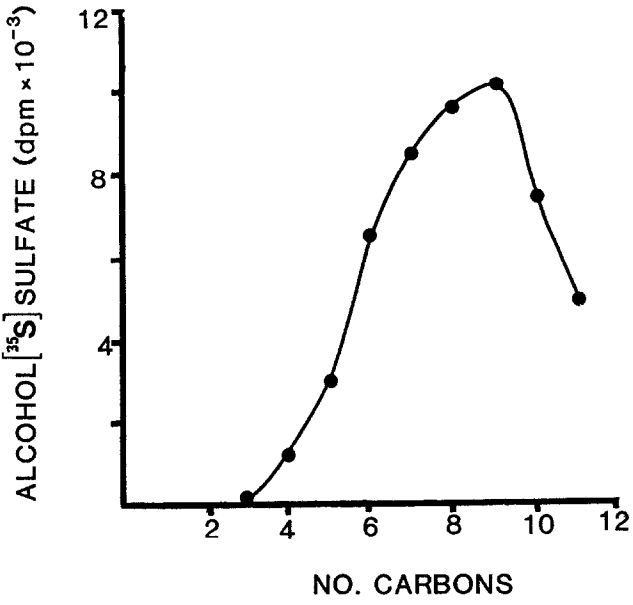

Fig. 1 Activity of human adrenal enzyme with the ascending series of normal aliphatic alcohols as substrates. All substrates were tested at 10 mM concentration. Incubation time was 30 min.

Fig. 1 shows that human adrenal hydroxysteroid sulfotransferase is also able to sulfurylate aliphatic alcohols of chain length C_3 or greater. All alcohols were tested at the same concentration of 10 mM and under these conditions sulfurylation rate rose with increasing chain length up to C_9 and subsequently decreased. Alcohols up to n-heptanol were freely soluble at 10 mM, but haziness, indicative of incomplete solubility, was associated with alcohols from n-octanol onwards. Alcohols were added from stock solutions in propylene glycol and increasing the percentage of propylene glycol in the final incubation, or addition of dimethylsulphoxide, did not improve the solubility characteristics. Thus solubility problems may have contributed to the

drop in sulphurylation rate with decanol and undecanol. Propylene glycol itself, when tested at the concentration used as vehicle (0.33%, v/v), gave identical counts to the blank using water. Methanol and ethanol at 10 mM gave identical counts to a water blank. Benzyl alcohol was sulfurylated at a rate intermediate between n-pentanol and n-hexanol. Since n-nonanol was sulfurylated at the highest rate, it was of interest to test the effect of the introduction of an additional hydroxyl group at the opposing end of the molecule. 1,9-Nonanediol was completely soluble at 10 mM and its sulfurylation rate was very similar to that of n-nonanol.

Kinetic studies

In marked contrast to the wave-like kinetics exhibited with dehydroepiandrosterone as variable substrate (1,2,3), normal Michaelis-Menten kinetics were found with aliphatic alcohols. Binding of the alcohols was independent to that of 3'phosphoadenosine-5'-phosphosulfate (Fig. 2). Due to the very high K_m values found, and to the solubility problems associated with the higher alcohols, kinetic data were restricted to n-propanol, n-butanol and n-pentanol. Kinetic constants are given in Table 1. Varying 3'-phosoadenosine-5'-phosphosulfate at separate fixed concentrations of n-butanol, also gave normal Michaelis-Menten kinetics and the K_m for the nucleotide was 8.3 μM (data not shown).

Antibody inhibition

Antibody (IgG) raised in rabbits against pure enzyme gave a single line by the Ouchterlony double diffusion technique. Titration of the enzyme with antibody was carried out using protein A linked to Sepharose

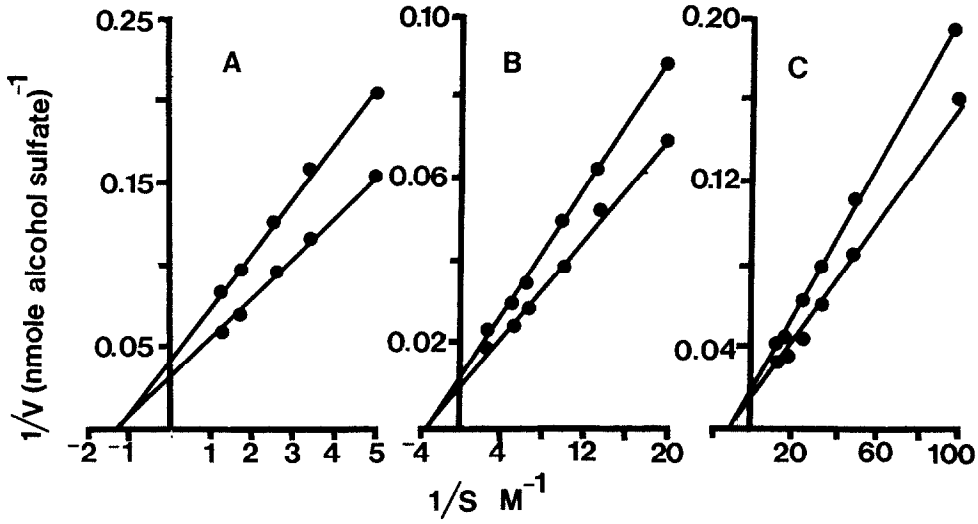

Fig. 2 Double reciprocal plots of kinetic data obtained with adrenal enzyme and aliphatic alcohols. A. n-propanol; B. n-butanol; C. n-pentanol. The alcohols were added from stock solutions in 0.05 M Tris/HCl, pH 7.5. Concentrations of 3'-phosphoadenosine-5'-phosphosulfate were 32 µM (upper line) and 76 µM (lower line), in each case. Initial velocities represent nmoles alcohol sulfate/min per mg protein.

TABLE 1. KINETIC CONSTANTS FOR ALPHATIC ALCOHOLS

Substrate	K_m	V_{max} nmole/min per mg protein
n-propanol	800 mM	33
n-butanol	277 mM	92
n-pentanol	133 mM	84
dehydroepiandrosterone	<1 µM*	∿62*

* Estimate only due to non-classical kinetics (1,2,3). The V_{max} is given as the maximum activity attained on varying dehydroepiandrosterone from 0-20 µM at constant 3'-phosphoadenosine-5'-phosphosulfate.

to remove enzyme-IgG complex, and dehydroepiandrosterone employed as substrate for residual enzyme activity. It can be seen from Table II that the amount of antibody required to reduce activity by 50% with this steroid as substrate was similarly effective when 17-epitestosterone and n-butanol were used as substrates.

TABLE II ENZYME ACTIVITY AFTER ADDITION OF ANTIBODY

Substrate	Residual activity (percentage of control)
Dehydroepiandrosterone	52
17-epitestosterone	59
n-butanol	55

Increasing amounts of antibody (IgG) to human adrenal hydroxysteroid sulfotransferase were added to aliquots of the enzyme (1.6 µg in 0.05 M Tris/HCl, pH 7.5, containing 0.1 mM dithiothreitol and 2% (v/v) propylene glycol) and allowed to stand at 25°C for 20 min. Protein A-Sepharose CL-4B suspension was then added in excess, and after a further 20 min at 25°C residual enzyme activity was assayed in the supernatant after centrifugation. Dehydroepiandrosterone was the substrate. The amount of IgG required to inactivate by 50% was then employed in a separate experiment and residual activity assayed with the 3 substrates.

Enzyme inactivation

It was established previously that whilst pure preparations of human adrenal enzyme retained activity for long periods in the frozen state, inactivation occurred upon incubation of the enzyme at 37°C. This could be prevented by the presence of the substrates (3). The enzyme preparation used in the present series of experiments proved to be stable when incubated at 37°C, but upon dialysis at 4°C against Tris-HCl buffer lacking thiol, inactivation occurred upon subsequent incubation at 37°C. This feature was used to further examine the

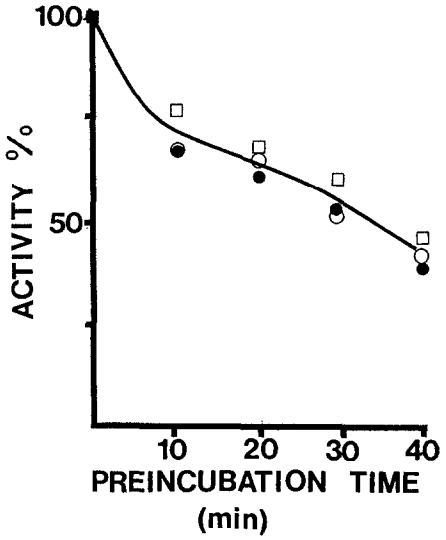

Fig. 3 Effect of preincubation of adrenal enzyme at 37°C prior to assay with substrates. The enzyme was routinely stored in 0.05 M Tris/HCl, pH 7.5, containing 0.1 mM dithiothreitol and 2% (v/v) propylene glycol, and held at -70°C. For this experiment an aliquot of enzyme was thawed and dialysed for 4 h at 4°C against 0.05 M Tris/HCl, pH 7.5, lacking thiol and propylene glycol. It was then preincubated at 37°C prior to assay. Dehydroepiandrosterone (●); 17-epitestosterone (□); n-butanol (○).

hypothesis that a single enzyme was responsible for sulfurylation of the wide spectrum of substrates outlined above. Enzyme activity for the three substrates dehydroepiandrosterone, 17-epitestosterone, and n-butanol was retained by prior incubation of the enzyme at 37°C. However, inactivation at similar rates occurred when the experiment was repeated with enzyme dialysed against buffer lacking thiol (Fig. 3).

Kinetics of dehydroepiandrosterone sulfurylation with bovine liver enzyme

The kinetics of steroid sulfurylation (1,2,3) and aliphatic alcohol sulfurylation (Fig. 2) are markedly different. Evidence presented in Fig. 3 and Table II indicate that one and the same enzyme is involved and this therefore suggests the presence of allosteric steroid-binding sites. To provide further support for this concept, it was decided to examine the kinetics of dehydroepiandrosterone sulfurylation by hydroxysteroid sulfotransferase isolated from bovine liver. This

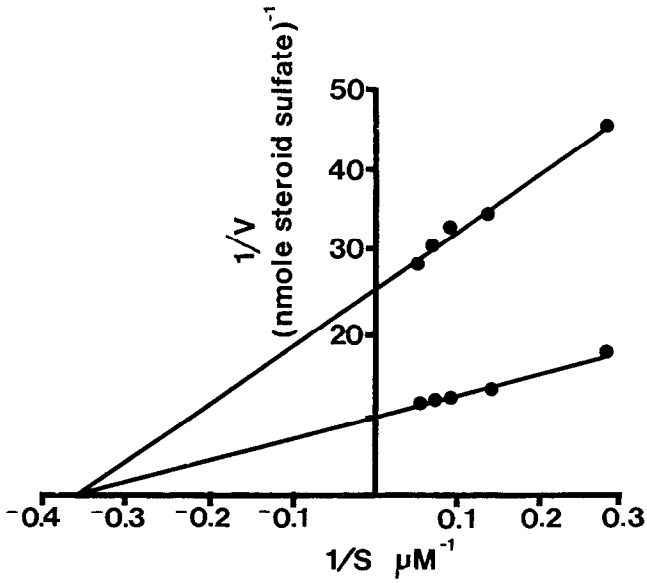

Fig. 4 Double reciprocal plots of kinetic data obtained with variable dehydroepiandrosterone and hydroxysteroid sulfotransferase purified from bovine liver. The concentrations of 3'-phosphoadenosine-5'-phosphosulfate were 52 μM (upper line) and 103 μM (lower line).

enzyme, isolated by affinity chromatography and DEAE-Sephadex chromatography, showed both the wide specificity and comparative rates of sulfurylation towards a group of steroids, as previously reported for the human adrenal enzyme (1,2). Again, like the latter enzyme, only monosulfates were formed from steroids containing two hydroxyl groups. However, the liver enzyme gave normal Michaelis-Menten kinetics with dehydroepiandrosterone as variable substrate, the K_m being 3 μM (Fig. 4).

DISCUSSION

Although the human adrenal enzyme is able to sulfurylate aliphatic alcohols of chain length C_3 or greater, the affinity is of a low order. An increasing capacity for sulfurylation of the ascending series of normal straight-chain alcohols was associated with a decrease in K_m (Table 1). With due regard to reservations imposed by solubility problems, n-nonanol was sulfurylated at the highest rate. It is of interest that molecular models (Drieden) of n-nonanol, in its fully extended form, bridged the length of the steroid rings from C_3 to C_{17}. One could perhaps envisage a fully extended conformation being imposed by binding within a hydrophobic pocket in the enzyme. The kinetic data in Table I would suggest that once bound, the aliphatic alcohols are sulfurylated at comparative rates to steroids.

Data presented in Table II and Fig. 3 strongly suggest that one and the same enzyme is responsible for sulfurylation of hydroxyl groups on C_3 and C_{17} of steroids, and also aliphatic alcohols. Such an interpretation is in keeping with the reported specificities of the sulfotransferases mentioned earlier, viz. estrone sulfotransferase and rat liver hydroxysteroid sulphotransferase. The latter enzyme possesses

different properties to the human adrenal enzyme, since the kinetics with variable steroid were presumably Michaelis-Menten, i.e. no mention was made that this was not the case (4). K_m values recorded for n-propanol, n-butanol and n-pentanol were 24, 3 and 1.7 mM, respectively, and thus much lower than those given in Table I. Methanol and ethanol were also sulfurylated (4). In addition, the amino acid compositions of the two enzymes are significantly different, particularly in the higher content of Try, Thr, Ser, Glu and Gly in the human adrenal enzyme. Finally, the rat liver enzyme exists as a hexamer of molecular weight 180 000 (4), compared to that of 68 000 for the human adrenal enzyme composed of 2 identical subunits (1). In our hands, the hydroxysteroid sulfotransferase isolated from bovine liver also exhibited normal Michaelis-Menten kinetics with dehydroepiandrosterone as variable substrate (Fig. 4).

The present data, implicating one enzyme in the sulfurylation of both C_3- and C_{17}-hydroxyl groups of steroids, confirms the suggestion made earlier (3) that steroid hormones can approach the sulfurylation site either via ring A, or via ring D after rotation of the molecule through 180°. Perhaps the most significant fact to emerge from the present studies relates to the normal kinetics obtained with simple alcohols, compared to the undulating initial velocity versus substrate plots given by dehydroepiandrosterone (1,2,3). When the latter is examined over a concentration range which might be construed as physiological, i.e. 0-1 μM, the activity rises initially then plateaus in the 0.5 to 0.8 μM region. At concentrations above 0.8 μM dehydroepiandrosterone, the rate increases markedly and this could provide a means of responding to sudden changes in levels of this steroid, for example

in response to ACTH action (3); actual levels of dehydroepiandrosterone in adrenal vein blood of individuals following ACTH administration range from 1-5 μM (9). Thus, the kinetic data obtained with simple alcohols provide further support for control of dehydroepiandrosterone sulfate secretion from the human adrenal via self-regulating dehydroepiandrosterone allosteric sites.

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